

THE REPRODUCTIVE PHYSIOLOGY OF WITCH FLOUNDER,  
*Glyptocephalus cynoglossus*

CENTRE FOR NEWFOUNDLAND STUDIES

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The reproductive physiology of witch flounder, *Glyptocephalus cynoglossus*.

By

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for the degree of Master of Science (Biology)

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## ABSTRACT

The witch flounder (*Glyptocephalus cynoglossus*), or grey sole, as it is commonly known, is a member of the family Pleuronectidae (right eye flounders) and is found in the Northwest Atlantic near Hamilton Bank off southern Labrador, southward over Newfoundland banks, Gulf of St. Lawrence, Scotian Shelf, Bay of Fundy and Gulf of Maine, to Cape Lookout, NC. The witch flounder has become increasingly important commercially since the 1940's. There was heavy exploitation of witch flounder by foreign ships in the early 1970's but with the introduction of Canada's 200-mile limit in 1977, foreign fishing for the species was reduced and Canada's take increased.

This study focused on the adaptation of wild witch flounder to captivity and the development of a captive broodstock. Areas of concentration focused on growth and maturation, with emphasis on the reproductive biology (pattern of oocyte development, endocrinology and gamete analysis) of the witch flounder. This information will be used to help determine whether the witch flounder is a good candidate as an aquaculture species.

The reproductive cycle of both the male and female witch flounder is characterized by distinct seasonal variations and fluctuations in plasma sex steroids associated with reproductive activity. As seen in other teleosts, the circulating levels of sex steroids increased as gamete maturation and gonad growth proceed, reaching peak levels during spawning.

Oocyte size-class frequency distributions of witch flounder demonstrate the presence of just a single clutch of progressively developing vitellogenic oocytes, indicating group-synchronous development, by far the most common reproductive strategy in teleosts.

Male witch flounder produce low volumes of viscous milt and sperm is only available for five months of the year (April – August). This correlates with spawning events in the female witch flounder, with ovulated eggs from late June to late August.

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## **CHAPTER 1.**

### **General Introduction.**

#### **1.1 THE NEED FOR AQUACULTURE**

As the world population continues to expand at an almost exponential rate, culture fisheries are becoming an ever more important source of food and resources. The natural stocks of fish can only supply a limited amount of food in a sustainable fashion. Overfishing, pollution, and habitat destruction have severely limited seafood populations worldwide and experts believe the current level of fishing may not be sustainable beyond the year 2040 (FAO 1996). Capture fisheries are the most widely known and recognized form of capturing aquatic organisms, and have been practiced since prehistoric times. Aquaculture, the cultivation of fish, shellfish and aquatic plants, is an established practice in many parts of the world (Fig. 1-1). Culture fisheries or aquaculture involves growing a selected organism, or in some cases several selected organisms, in a controlled environment where the sole purpose of the organisms is to be harvested and then sold commercially. Most aquaculture crops are destined for human consumption. However, in the case of salmon, government agencies use their product to enhance the declining natural resources. Other products include baitfishes, ornamental and aquarium fish and aquatic animals used to increase natural populations for capture and sport. Aquaculture farms are very similar to their land based counterparts in terms of concept and management strategies.

One of the advantages of cultured product over commercially harvested fish is the quality. Cultured fish suffer minimal handling and reach the market without delay, resulting in a fresher product. In fact, an increasing number of fish species are being sold live. In addition,

cultured products are not subject to seasonal shortages like commercially harvested fish, but are accessible year round, resulting in more stable prices (Bidwell 1999).

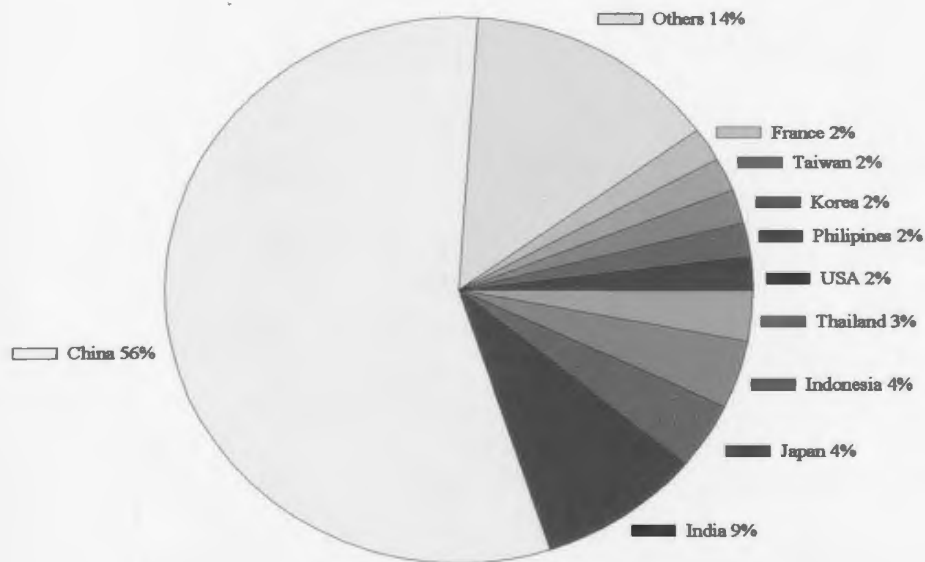


Fig 1-1. Top 10 Aquaculture Producers (By volume in 1994) (FAO 1996)

## 1.2 CANADIAN AQUACULTURE

In Canada, aquaculture was first used to enhance natural stocks; however, it is now a large-scale commercial industry across the country providing direct and indirect economic benefits to many local and regional economies. All ten provinces and the Yukon Territory currently have a stake in commercial aquaculture and interest is increasing in the Northwest Territories. Aquaculture production in 1998 accounted for 27% of total landed value of Canadian fish and seafood (DFO 2000). Commercial aquaculture production dates to the



1950s, when trout and oysters were the species of interest. Over the past 20 years, commercial production has expanded to include several salmon species, mussels, clams, oysters and scallops.

### **1.3 THE AQUACULTURE INDUSTRY IN NEWFOUNDLAND**

Since the dramatic collapse of the commercial fishery off the east coast of Canada in 1993, interest in developing marine finfish aquaculture in Atlantic Canada has increased. The most promising solution for relieving the fresh fish deficit and likewise providing jobs for out-of-work fishermen is aquaculture. The Canadian government has increased its support for aquaculture research in the years since the fishery moratorium began in the Atlantic Provinces and the result has been the development of an aquaculture industry in Newfoundland. In 1998, the total export value for aquaculture in Newfoundland and Labrador is estimated at approximately \$11 million compared to \$4 million in 1995, and employed more than 400 people in 2000 (Table 1-1). There were also 125 licensed aquaculture sites: 68 commercial shellfish licenses (blue mussels and scallops) and 57 commercial finfish licenses (steelhead trout, Atlantic salmon, rainbow trout, arctic char, and cod) (Fig. 1-2). Research continues on mussels, scallops, haddock, Atlantic cod, yellowtail and witch flounder. There is an interest in halibut farming in the Atlantic provinces as the world demand for this premium product is high and the wild supply is low (DFO 1999). Witch flounder were considered an attractive species for culture given their high value in the market and low supply from the wild fishery.

**Table 1-1: Newfoundland and Labrador Aquaculture Statistics 1998 (DFO 1999)**

<b>Type of Fish or Shellfish</b>	<b>Tonnes</b>	
	<b>Produced (mt's)</b>	<b>Export Value (\$000)</b>
<b>FINFISH</b>		
Salmon	401	2,925
Trout	48	197
Steelhead	1,316	6919
<b>TOTAL FINFISH</b>	<b>1,765</b>	<b>10,041</b>
Blue Mussels	946	815
Scallops	9	53
Other	7	32
<b>TOTAL SHELLFISH</b>	<b>1,051</b>	<b>900</b>
<b>GRAND TOTAL</b>	<b>2,724</b>	<b>10,941</b>

#### **1.4 GENERAL BIOLOGY OF WITCH FLOUNDER**

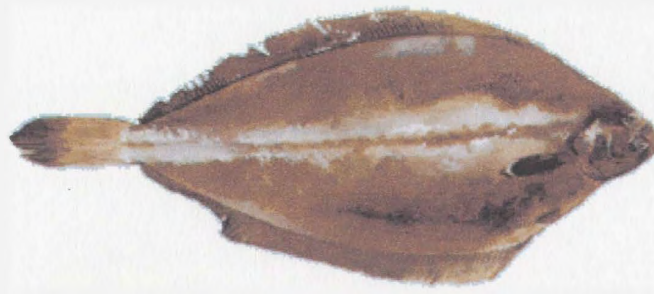
The witch flounder (*Glyptocephalus cynoglossus*), or grey sole (Fig. 1-3), as it is commonly known, is a member of the family Pleuronectidae (right eye flounders) and is found in the Northwest Atlantic near Hamilton Bank off southern Labrador, southward over Newfoundland banks, Gulf of St. Lawrence, Scotian Shelf, Bay of Fundy and Gulf of Maine, to Cape Lookout, NC. Under international quota regulations in 1974, the fishery was divided

into five different stock areas for management purposes. The five stock areas are: (1) southern Labrador-eastern Newfoundland (2J-3KL), (2) southern Grand Banks (3NO), (3) St.Pierre Bank-Fortune Bay (3P), (4) northern Gulf of St.Lawrence (4RS) and (5) Scotian Shelf (4VWX) (Underwater World 1983).



**Fig 1-2. Aquaculture sites in Newfoundland and Labrador (1999) (DFO 1999)**

Witch flounder are generally deepwater flatfish living mainly at depths of 45-274 m, but may also be found between 18 and 1570 m. It prefers a mud-sand bottom and in summer they usually move up onto the soft mud and in winter move down into the deep gullies. Witch flounder have been caught in a bottom temperature range of -1°C - 11°C, however they are more abundant at temperatures of 2-6°C (Scott & Scott 1988).



**Fig. 1-3. Witch Flounder , *Glyptocephalus cynoglossus* (Fishbase 2000)**

The witch flounder is dark greyish brown on the eyed side and white on the underside. The body is covered by smooth scales, which make it very slippery and extremely difficult to hold. It lies on its left side with the stomach and other visceral contents on the right. The body is relatively narrow when compared to other flatfishes and has a very small head (1/5 of the total body length) with a very small mouth not unlike the yellowtail flounder. It can grow as large as 78cm in length with a weight of 3.5-4.0 kg but generally witch flounder beyond 60cm in length and 2.5kg in weight are uncommon.

Witch flounder is relatively slow growing; the fastest growth rate occurs in the northeast Newfoundland shelf area with the slowest growth rate in the Gulf of St. Lawrence area. They are a long-lived (30 year or more) species, with females living longer than males. Unlike most marine fish species where size at age is greater in the more southerly areas, the opposite is true of witch flounder for both males and females.

It is a predaceous species, limited in choice in food items by its small mouth. Principal food items include polychaets, including tubeworms, and crustaceans (particularly

amphipods), small fishes and mollusks such as small bivalves. During spawning season these fish do not feed much, if at all. Size at sexual maturity is smaller for males than for females. Spawning usually takes place in very deep water and occurs over a prolonged period, extending from March to September in the northwest Atlantic. In southern Labrador-Newfoundland shelf region spawning occurs from March to July but most intensively from March to May. Spawning on the Grand Bank region occurs principally in July and August while spawning on the Georges Bank-Scotian Shelf region occurs from May to possibly October, reaching its peak in July and August (Scott & Scott 1988). A female 45cm long from the Grand Bank produces about 200,000 eggs annually, a female 55cm long about 450,000 eggs. Eggs are spherical, 1.10 - 1.45 mm in diameter, are pelagic but without an oil globule. Fertilized eggs float; hatching occurs in about 7-8 days at 8°C.

Young flounder are 4-6 mm long on hatching and may remain floating about, in a pelagic state, for upwards of a year before settling down on the bottom, the longest pelagic stage of any North Atlantic flatfishes, such as yellowtail flounder, winter flounder, plaice and halibut (Rabe 1999).

## **1.5 COMMERCIAL SIGNIFICANCE OF WITCH FLOUNDER**

The witch flounder has become increasingly important commercially since the 1940's. There was heavy exploitation of witch flounder by foreign ships in the early 1970's but with the introduction of Canada's 200-mile limit in 1977, foreign fishing for the species was reduced and Canada's take increased. They are taken incidentally as a by catch when fishing for other species such as cod, halibut, plaice and redfish. Commercially caught witch flounder are usually 8-13 years old, weigh ~0.7 kg and are ~45 cm long. The flesh is white

and considered to be of high quality in flavor and texture. It is usually marketed, fresh or frozen, as fillets of sole and when compared with the other small flounders, they command a higher price and sometimes more than halibut (Table 1-2). Worldwide, the US harvests between 2,000 and 2500 tones of witch flounder per year and the European production is estimated at 15,000 to 20,000 tons per year (Library 2000). In 1989, 7, 272 metric tonnes of witch flounder were caught in Atlantic Canada whereas in 1999 only 1,109 metric tonnes were caught, following the decline that most fisheries are experiencing.

## **1.6 OBJECTIVES OF RESEARCH**

The development of new candidate finfish species from the northwest Atlantic for aquaculture depends upon the control of many biological and physical environmental factors. It should be biologically manageable, have suitable growth profile, realistic promise of financial return and have existing markets and routes of commercialization identified (Barnette 1998). There are three strategies for aquaculture start-up: 1) start with stocks of juveniles collected from the wild, 2) start with viable gametes produced by adult fish from the wild and 3) start with developing a domesticated broodstock. Most importantly, it is essential to have a predictable source of offspring (Crim and Wilson 1998).

Witch flounder have rarely been held in captivity so there is little information about its reproductive activities. This study focused on the adaptation of wild witch flounder to captivity and the development of a captive broodstock. Areas of concentration will be focused on growth and maturation, with emphasis on the reproductive biology (pattern of oocyte development, endocrinology and gamete analysis) of the witch flounder. This information



will be used to help determine whether the witch flounder is a good candidate as an aquaculture species.

**Table 1-2: Comparison prices for flatfish species (US\$) (Fulton Fish Market 2000)**

SPECIES	COMMON NAME	PRICE (lb)
<i>Glyptocephalus cynoglossus</i>	Grey sole	\$4.00
<i>Pleuronectes ferrugineus</i>	Yellowtail	\$2.00
<i>Pleuronectes americanus</i>	Blackback	\$1.48
<i>Hippoglossoides platessoids</i>	Plaice	\$1.51
<i>Hippoglossus hippoglossus</i>	Halibut	\$5.27

### **1.6.1 Growth, maturation and ovarian development**

Normally, the conditions for holding newly acquired broodstock closely emulate the field situation, with similar light and oxygen levels, thermal regimes and feeding schedules. But for reasons of either convenience or practicality, broodstock usually will be acclimated to possible stressful rearing conditions, which may negatively impact reproductive performance. While most fish species appear capable of undergoing gonadal development in captivity, few are able to spontaneously spawn viable gametes (eg. Atlantic cod). Therefore, considerable research is aimed at developing gamete collection and fertilization protocols for captive broodstock.

Fish respond to changes in the environment, and reproduction is timed to coincide with environmental conditions that are most favorable for reproduction. In most species gonad growth is commonly influenced by changes in temperature and photoperiod because collectively, they are reliable cues, which foretell the advent of the appropriate time for spawning. Close to the time of gamete final maturation, appropriate physical cues (such as water flow or level, availability of suitable physical space and availability of spawning substrate) are often required for ovulation and/ spawning (Stacey 1984).

Ovarian development can be classified into 3 basic types, 1) synchronous – species that spawn once and then die such as Pacific salmon, are characterized by a single clutch of oocytes that grow in unison. 2) Group synchronous and multiple group synchronous – at least two clutches of oocytes can be distinguished in the ovary at the same time, hence these species spawn more than once. Group synchronous species spawn more than once in a lifetime, but typically once per season whereas multiple group synchronous species have multiple spawning episodes within a single reproductive season. 3) Asynchronous – in these species,

oocytes are a mixture of stages and no distinct clutches can be identified, although asynchronous development is most likely an extreme case of multiple group synchrony (Pankhurst 1998).

### ***1.6.2 Endocrinology of female and male witch flounder***

In order for reproduction to occur in captive fish, they have to undergo gonadal development and maturation, and gamete final maturation resulting in ovulation in females (release of eggs into the oviduct) and sperm production in males (Pankhurst 1998).

Bottlenecks can occur at any of these stages, possibly as a result of inappropriate environmental conditions (social and physical) and/or chronic stress imposed by the conditions of captivity (Pankhurst 1998). Controlled approaches to managed reproduction rely on a solid understanding of the pattern of gamete development, duration and frequency of spawning events and associated endocrine changes. Armed with this information, potential bottlenecks can be identified and management strategies implemented.

The role of steroids testosterone (T) and estradiol – $17\beta$  ( $E_2$ ) is standard among teleost species. T is a precursor to  $E_2$  (Matsuyama et al 1988) and  $E_2$  stimulates synthesis of the yolk precursor vitellogenin (Vtg), which is incorporated into the growing oocyte during vitellogenesis (Speckler and Sullivan 1993). As vitellogenesis approaches completion there is a fall in  $E_2$ , then T and a surge in the production of  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one, which is the maturation steroid on most species in which it has been investigated (Scott and Canario, 1987).

In male teleosts, plasma T levels tend to be highest during spermatogenesis (transition from spermtogonia to spermatids) and tend to drop off prior to spermiation (sperm

release) (Harmin et al., 1995; Carolsfield et al., 1996). In many species 11-ketotestosterone (11-KT) is elevated during spermatogenesis and the early stages of spermiation, and is thought to be more effective than T at stimulating spermatogenesis, secondary sexual characteristics and spawning behavior (Borg 1994).

### **1.6.3 Sperm Analysis**

The pattern of male gamete development broadly reflects gamete synchrony shown in females (Pankhurst 1994). Milt is often present in the sperm duct one to two months earlier than the beginning of the spawning season in females, for example in starry flounder, *Platichthys stellatus* (Pallas) and plaice, *Pleuronectes platessa* L. (Barr 1963). Winter flounder males produce sperm five months longer than the female spawning season (Burton and Idler 1987) and in some species like the dab, *Pleuronectes limanda* L., and the yellowtail flounder, *Pleuronectes ferrugineus*, sperm is present throughout the year (Clearwater and Crim 1998).

Like most other externally fertilizing marine teleosts, witch flounder sperm is immotile when collected from the urogenital pore and is activated upon dilution in seawater (Morisawa and Suzuki, 1980). Changes in osmotic pressure, pH and ionic concentrations are thought to be the most important factors in triggering sperm activation in teleost fish (Billard et al., 1992). Witch flounder males produce low volumes of sperm (200-350 $\mu$ l); therefore in order to manage male broodstock effectively, it is important to understand milt characteristics in order to effectively manage the low sperm volumes.

## 1.7 THESIS STRUCTURE

- Chapter 2 – Focuses on the acclimation of captive witch flounder to laboratory conditions with an emphasis on growth and maturation
- Chapter 3 – Focuses of the reproductive endocrinology of male and female witch flounder
- Chapter 4 – Focuses on the biochemical characteristics of the milt of the male witch flounder.
- Chapter 5 - Summary and Conclusions

## 1.8 REFERENCES

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## **CHAPTER 2.**

### **Capture/growth, Maturation and Oocyte Development.**

#### **2.1 INTRODUCTION**

The development of a new candidate fish species for cool-water aquaculture depends upon control of many factors, including a predictable source of offspring. While aquaculture projects may be started with founder stocks of juveniles or gametes collected from the wild, a secure supply of offspring can only be assured by adapting and manipulating captive broodstock to perform well under culture conditions (Crim and Wilson 1998).

The physical injury and physiological stress of capturing, handling, transporting, injecting and holding brood fish can have a greater detrimental effect on spawning success than almost any other set of factors. Fish must be handled carefully and optimum water conditions must be maintained to minimize stress. The conditions for holding newly acquired broodstock must closely emulate the field situation, with similar light and oxygen levels, thermal regimes and feeding schedules.

Oogonia in females develop into pre-vitellogenic oocytes, and at the acceleration of oocyte growth, the phospholipoprotein yolk precursor vitellogenin (Vtg) is synthesized in the liver and subsequently taken up by developing oocytes (Tyler and Sumpter 1996). At the completion of vitellogenesis, oocytes undergo final maturation, which is characterised by the disintegration of nuclear membrane (germinal vesicle breakdown). In association with this, there is generally coalescence of lipid droplets and yolk to give the cytoplasm a homogeneous and often hyaline appearance (Nagahama 1983). In marine species with pelagic eggs there is a final increase in size as the oocyte hydrates and this may be accompanied by formation of one or more oil droplets (Wallace and Selman 1981).

There are generally three commonly recognized modes of oocyte development. In species such as anguilled eels or Pacific salmon, which spawn once and then die, there is a single clutch of maturing oocytes present in the ovary. This is described as synchronous ovarian development (Wallace and Selman 1981). Group synchronous development describes species that spawn more than once in their lifetime but typically only once per season. In this case, the ovary contains both a population of previtellogenic oocytes and the developing clutch destined for maturation and ovulation for the current spawning season. This type of development is found in cold temperate species such as trout (Tyler et al. 1990), deepwater marine species (Pankhurst et al. 1987) and high latitude marine species (North and White 1987). In some species there is more than one developing clutch present in the ovary and is characteristic of species where there are multiple spawning events within a single reproductive season.

For reasons of convenience or practicality, broodstock are generally acclimated to extraordinary and possibly stressful rearing conditions, which may negatively impact reproductive performance. While most fish species appear capable of undergoing gonadal development in captivity, few are able to spontaneously spawn viable gametes. Considerable research is aimed at developing gamete collection and fertilization protocols for captive female broodstock.

In this study, we attempted to capture witch flounder, transport them back to the lab and have them acclimate to lab conditions as potential broodstock. Surviving fish were then monitored for growth and maturation.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 *Broodstock collection***

Adult female and male witch flounder were caught by Danish seine fisherman, in Fortune Bay, Newfoundland at a depth of 260 meters. From the time the fish were brought on board the trawler, they were carefully handled and maintained in three plastic tubs with cool, flow through sea water from a deck hose. The trip to shore lasted one hour. Once on land, fish were transferred to a tank inside a cargo truck for a 9-hour drive to the Ocean Sciences Center, Logy Bay, Newfoundland. Temperature and oxygen concentration was monitored and recirculating pumps were used to increase oxygen availability when the oxygen concentration fell below 80%. Collections were made in November 1997 and July 1998.

### **2.2.2 *Holding conditions***

Upon arrival at the lab the fish were injected with 100µl/kg of an antibiotic (Trivetin, Pitman-Moore Company, Ontario, Canada) and kept quarantined in a tank with ambient flow-through sea water and minimum disturbance. During the first week, the fish were treated with Chloramine T, (Syndel Laboratories, Vancouver, BC, Canada) 5mg/L for one hour 3 times over a one-week period. After two weeks, the surviving fish were individually tagged with a passive integrated transponder (PIT) (BioSonics, Seattle, Washington, USA) and moved to the experimental tank. The fish were held in 200-liter fiberglass tanks with flow-through sea water at either ambient temperatures or in a mixture of ambient and chilled or heated sea water. Daily water temperatures were recorded from May 1998 through August 1999 by temperature

monitors (Radio Shack, Canada). Water temperatures ranged from as low as 3°C in the winter months to as high as 16°C during the summer (Fig. 2-1). Fish were exposed to a simulated natural photoperiod for St. John's, Newfoundland (47°20'N, 52°45'W) from minimal indirect lighting (10 Lux). Fish were fed to satiation twice weekly on a moist shrimp-based formulated pellet (Table 2-1).

### ***2.2.3 Oocyte Size-Frequency Distributions***

After the initial two week quarantine period, while the fish were being sorted into the experimental tanks, small amounts of ovarian tissue was collected from fresh mortalities and fixed in 0.6% NaCl containing 1% formalin (Harmin 1991) for a minimum of 2 days before observations were made. Individual oocytes from the fixed tissues were separated under the microscope using fine forceps. Previtellogenic and vitellogenic oocytes could be easily distinguished by their size and appearance, and 100 vitellogenic eggs were measured by optical micrometer to the nearest 26µm under an Olympus dissecting microscope at 40X magnification. The size-class frequency was determined from these measurements.

### ***2.2.4 Determination of Gonadosomatic Index***

At the time of autopsy, body weight and length, as well as gonad weight, were recorded for calculation of gonadosomatic index (GSI) = ((gonad weight / body weight) x 100). All fish autopsied resulted in three different GSI types. For purpose of discussion they were arbitrarily classified as follows: 1) immature (GSI < 1%), 2) maturing (GSI 1.1% < 8%) and 3) ovulating (GSI >10%).



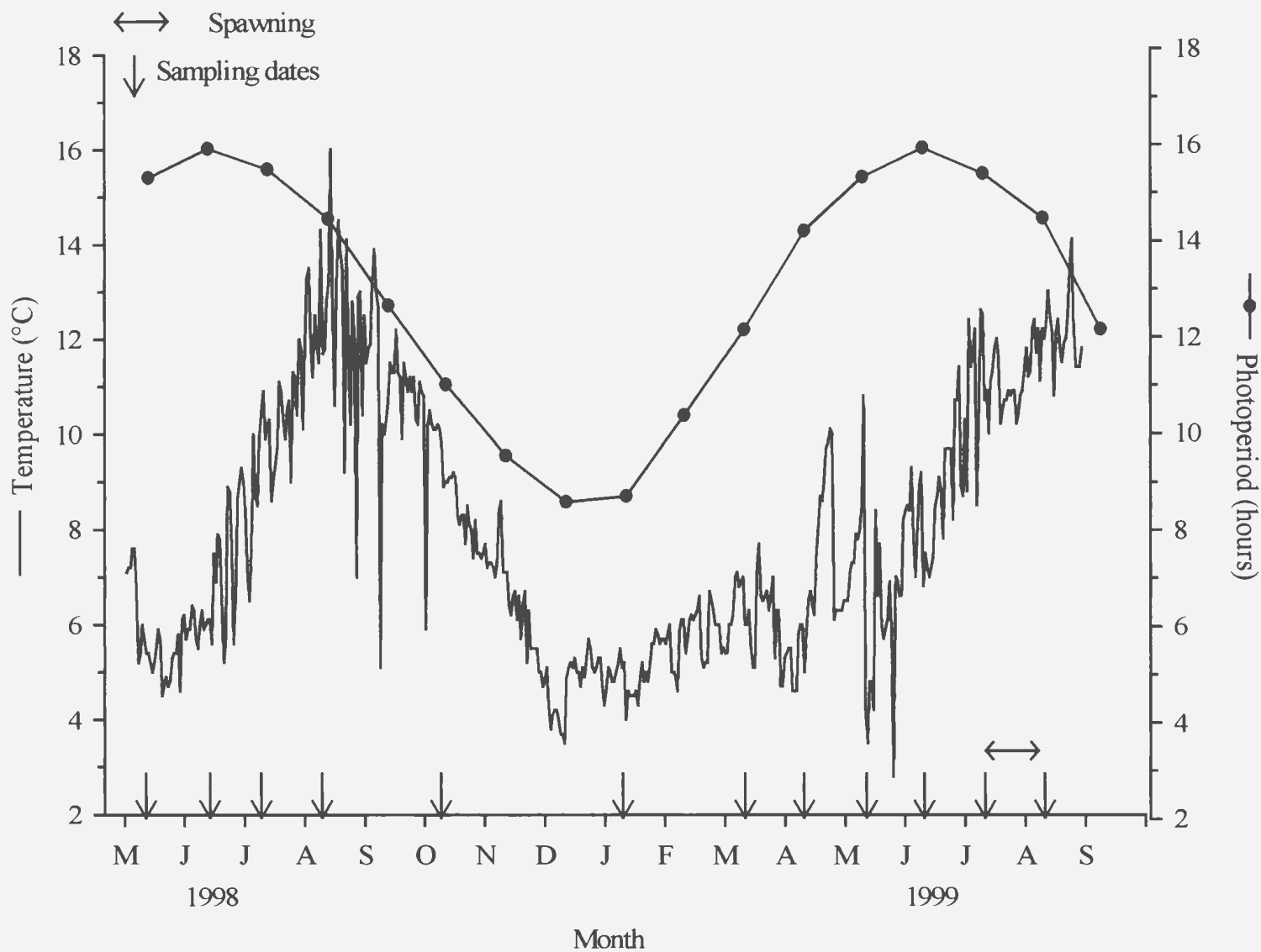


Fig. 2-1: Water temperatures (°C) and photoperiod (hours) for captive witch flounder, *Glyptocephalus cynoglossus*.

( $\longleftrightarrow$ ) Indicates duration of female spawning and ( $\downarrow$ ) indicates sampling dates

**Table 2-1: Moist pellet diet formulation for witch flounder broodstock**  
(NRC, Halifax)

Ingredient	Purpose	Company	g/kg
Finely ground shrimp	protein	Local <sup>1</sup>	450
Herring meal	protein	Corey Feed Mills <sup>2</sup>	290
Sapropeche (CPSPG)	protein	Surr Gain <sup>3</sup>	40
Wheat middlings	carbohydrate	Corey Feed Mills	141
Pre-gelatinized starch	binder	National Starch and Chemical Corporation <sup>4</sup>	40
Liquid krill extract	gelatin	BDH <sup>5</sup>	5
Choline chloride		Corey Feed Mills	4
Vitamin premix	vitamins	Conners Brother Ltd <sup>6</sup>	10
Mineral premix	minerals	Conners Brothers Ltd.	5
Herring oil		Corey Feed Mills	15

1- Seafood Shop, Churchill Park, St.John's, NF A1B 4R5

2- Corey Feed Mills, 136 Hodgson Rd., Fredericton, NB E3B 8W6

3- Shur Gain, 494 Willow St., Truro, NS B2N 5G7

4- National Starch and Chemical Corporation, FINDERNE Avenue,  
P.O.Box 6500, Bridgewater, NJ 08807

5- BDH Inc., 350 Evans Avenue, Toronto, ON M8Z 1K5

6- Conners Brothers Limited, Black Harbour, NB E0G 1H0

### **2.2.5 Monthly Sampling**

Beginning May 1998 and terminating on August 1999, the surviving fish were measured (15 times). Individual body weight (g) and length (cm) were recorded. Condition factor index (CF) ( $CF = (\text{body weight}/\text{body length}^3) \times 100$ ) was calculated for each fish. Each fish was checked for milt production and a blood sample was taken from every fish. Females were identified by the observance of vitellogenic oocytes in the ovary. Oocytes were gently aspirated from the anterior of the right (eyed side) ovary by inserting a polyethylene cannula with an inner diameter of 1.9mm through the ovipore (Larsson et al. 1997). Gentle pressure was applied to the abdomen of the fish and males were identified by the presence of milt from the urogenital pore.

### **2.2.6 Maturation**

Ovulation of female witch flounder was checked every second day, beginning early July 1999, by applying slight pressure to the swollen abdomen in the direction of the egg pore. Freshly ovulated eggs from individual females were collected in pre-chilled 100ml beakers and placed on ice. Milt used in the fertilization trials was collected from two males at the same time as egg collection. Milt, which was manually stripped by applying gentle pressure posterior to the urogenital pore, was aspirated into pre-chilled tuberculin syringes and stored on ice. Care was taken to avoid contamination of the milt sample with seawater or urine. Individual samples were checked for motility and only the samples of highly motile sperm

were used. After the major ovulation event, checking was continued twice more in two-day intervals, and no more eggs were collected.

### **2.2.7 *Egg Quality***

Egg quality has been defined by morphological criteria - egg viability, fertilization and hatching rates. Each batch of eggs collected from individual females was evaluated. High-quality eggs (good viability) from marine teleosts with pelagic eggs are generally clear, floating, and spherical, and have no previtelline space prior to fertilization (McEvoy 1984 and Larsson et al 1997).

#### **2.2.7.1 Egg Viability**

For egg viability determinations, three aliquots of unfertilized eggs, per female were mixed with sea water. One hundred eggs were observed from each sample and the number of clear, floating, and spherical eggs lacking a previtelline space was noted.

#### **2.2.7.2 Fertilization**

Fertilization trials were conducted in petri dishes on ice, by mixing 75µl (~150 eggs) of eggs with 10µl of milt and then 200µl of seawater was added. This preparation was mixed again and left for 2 minutes after which 20mls of seawater was added to the petri dish and the dish was placed in a dark incubator (Hotpack Corp, Model # 352602, Philadelphia, PA 19154) set at 5°C. Three replicates of each female were tested.

Approximately 15 hours after fertilization, the numbers of fertilized eggs were counted by observing the 4-32 cell stage of development under an Olympus dissecting microscope at 16X magnification. Fertilization rates were calculated from the numbers of fertilized eggs out of the total number of viable eggs in the dish. Every two days after fertilization, dead eggs (opaque eggs showing no embryo development) and hatched larvae were counted and removed from the petri dish and the seawater was changed. This procedure continued until all the eggs had either hatched or died. The seawater used in the fertilization and hatching experiments was filtered, UV sterilized, and supplemented with antibiotics (30 mg l<sup>-1</sup> penicillin G, 50 mg l<sup>-1</sup> streptomycin sulphate).

#### 2.2.7.3 Hatching Rates

Hatching rates were determined by the total number of hatched larvae expressed as a percentage of fertilized eggs.

#### 2.2.7.4 Estimation of Egg Production

Egg batches were measured in a cold, clean, graduated 100 ml cylinder, and the volume recorded to the nearest milliliter. Triplicate batch fecundity estimates were done by pipetting aliquots of 200µl. After counting the eggs under a dissecting microscope, a mean of the triplicates was calculated and was converted to an egg concentration value (eggs per ml). Batch fecundity was calculated by multiplying egg volume by the mean egg concentration. Total egg production was determined from the sum of the batch fecundities.

### **2.2.8 *Statistical Analysis***

Data were analyzed using the non-parametric Wilcoxon Signed Ranks Test ( $p < 0.05$ ) using the SPSS computer package. Data were log, square root or arcsine transformed when necessary to fit the assumptions of normality. Data are expressed as means  $\pm$  standard error.

## **2.3 RESULTS**

### **2.3.1 *Survivability and animal selection***

In November 1997, over two hundred animals were transported into the laboratory and less than 15% survived. By the beginning of the experiment (May 1998) only twenty-five fish were still alive. Of these fish, twelve were identified as females and six were confirmed males. Females were determined by the observance of vitellogenic oocytes (yellow in color- indicating the presence of yolk) in the ovary. Oocytes were gently aspirated from the anterior of the right (eyed side) ovary by inserting a polyethylene cannula with an inner diameter of 1.9mm through the ovipore (Larsson et al 1997). Gentle pressure was applied to the abdomen of the fish and males were identified by the presence of milt from the urogenital pore. These are the fish that were followed through the experimental time period. Approximately 120 fish were collected in July 1998, however none of these fish survived. The majority of these fish were mature females that would have likely spawned in the near future.

### ***2.3.2 Growth – Males***

Male body weight (  $F = 3.502$ ,  $p < .001$ ) and CF (  $F = 2.544$ ,  $p < .009$ ) showed significant monthly changes during the experimental period. Weight increased from an average of 450 gm in May 1998 to over 600 gm in August 1999. CF increased from 1.0 % to 1.25 % over the same period (Fig. 2-2).

### ***2.3.3 Growth – Females***

Four out of the twelve females matured and produced viable eggs. These fish were also significantly larger ( $F = 2.278$ ,  $p < .002$ ) than the remaining non-spawning females. For the purpose of this study the two groups will be discussed separately.

### ***2.3.4 Growth – Spawning females***

The body weight ( $p < 0.866$ ) and CF ( $p < 0.962$ ) of the spawning females showed no significant monthly changes for the experimental period. The average body weight increased from 825g in May 1998 to 900g in July 1999. However, there was a decrease in weight in August, after the spawning period. Notably, the average post-spawning weight was unchanged from the beginning of the study (Fig. 2-3).

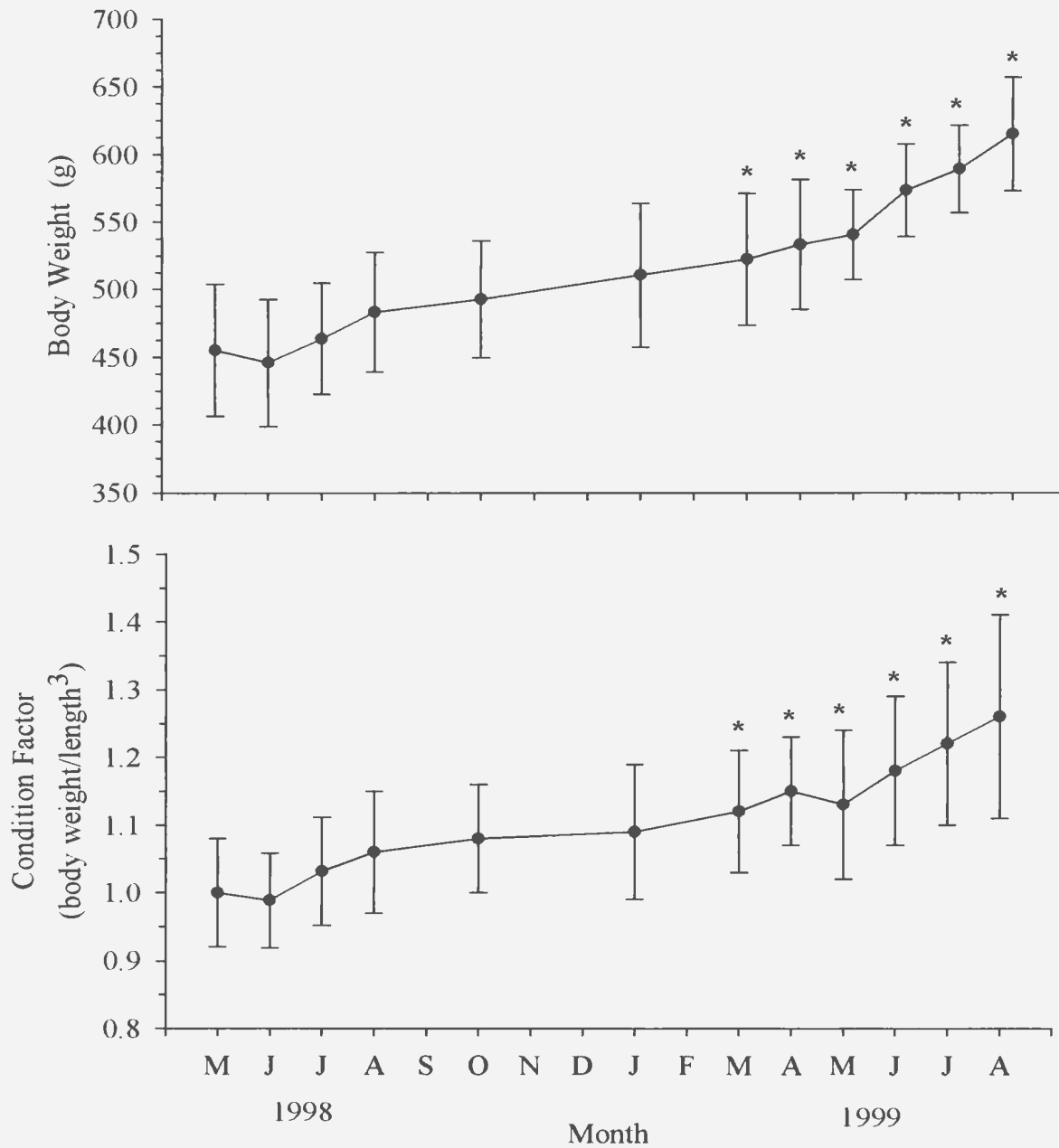


Fig. 2-2: Mean body weight (g) changes (Panel A) and mean CF (%) changes (Panel B) in male witch flounder from May 1998 to August 1999. Error bars represent standard errors (n=6).

Means with an asterisk (\*) were significantly different from the initial time period (Wilcoxon Signed Ranks Test,  $p < 0.05$ ).



### **2.3.5 Growth - Non-spawning females**

Non-spawning female body weight ( $p < 0.012$ ) and CF ( $p < 0.036$ ) showed significant monthly changes for the duration of this experiment. The average body weight was 515g in May 1998 and increased to an average of 580g in August 1999. CF ranged from 1.12% in May 1998 to 1.25% in August 1999 (Fig. 2-4)

### **2.3.6 Ovarian biopsies**

Oocyte distributions from mortalities from fish collected in November 1997 demonstrated only GSI types 1 & 2, while samples collected in July 1998 reflected only GSI type 3 (Fig 2-5). The ovary of immature females (type 1) contained only vitellogenic oocytes with diameters, which ranged from 250 $\mu$ m to 450 $\mu$ m. Type 2 or maturing females consisted of ovaries with two clearly distinguishable clutches of oocytes. Vitellogenic oocytes made up 90% of the biopsy sample and the remainder oocytes were previtellogenic. The diameters of the vitellogenic oocytes ranged from 850 $\mu$ m – 1040 $\mu$ m. Ovulating females exemplify only type 3 GSI's. All oocytes measured were hydrated and clear. The oocyte diameters ranged from 1120 $\mu$ m – 1350 $\mu$ m. Estimates of GSI's were hard to obtain as oocytes were loosely contained within the body cavity.

### **2.3.7 *Egg production***

Of the twelve females in this study only four females produced viable eggs in 1999. Only one batch of eggs per female was released. The range of body weight varied from 596g to 973g.

#### **2.3.7.1 Egg volumes**

Egg volumes fluctuated from as low as 37mls to as high as 77mls (Table 2-2). Once egg volume is correlated to body weight, the fecundity of these females varied from 44,884 eggs/kg – 57,136 eggs/kg, with an average of 50,099.5 eggs/kg (Table 2-3).

#### **2.3.7.2 Viability**

Viability rates varied from a low of 67% to a high of 90%. Numbers reflect an average of three replicates  $\pm$  standard error (Table 2-3).

#### **2.3.7.3 Fertilization Success**

Fertilization rates ranged from 71% to 88%. Numbers reflect an average of three replicates  $\pm$  standard error (Table 2-3).

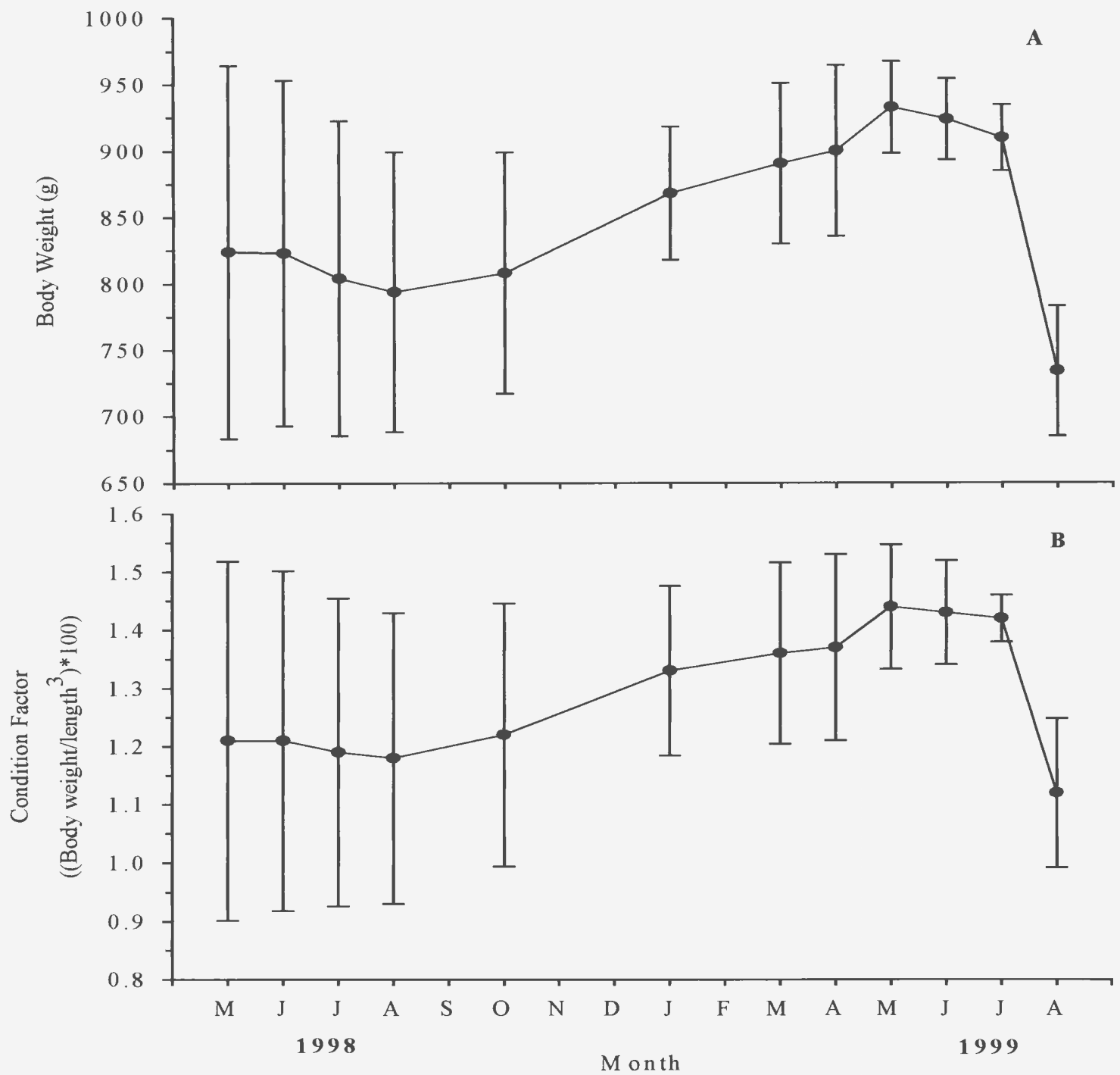


Fig. 2-3: Mean body weight (g) changes (Panel A) and mean CF (%) changes (Panel B) in spawning female witch flounder from May 1998 to August 1999 (n=4).

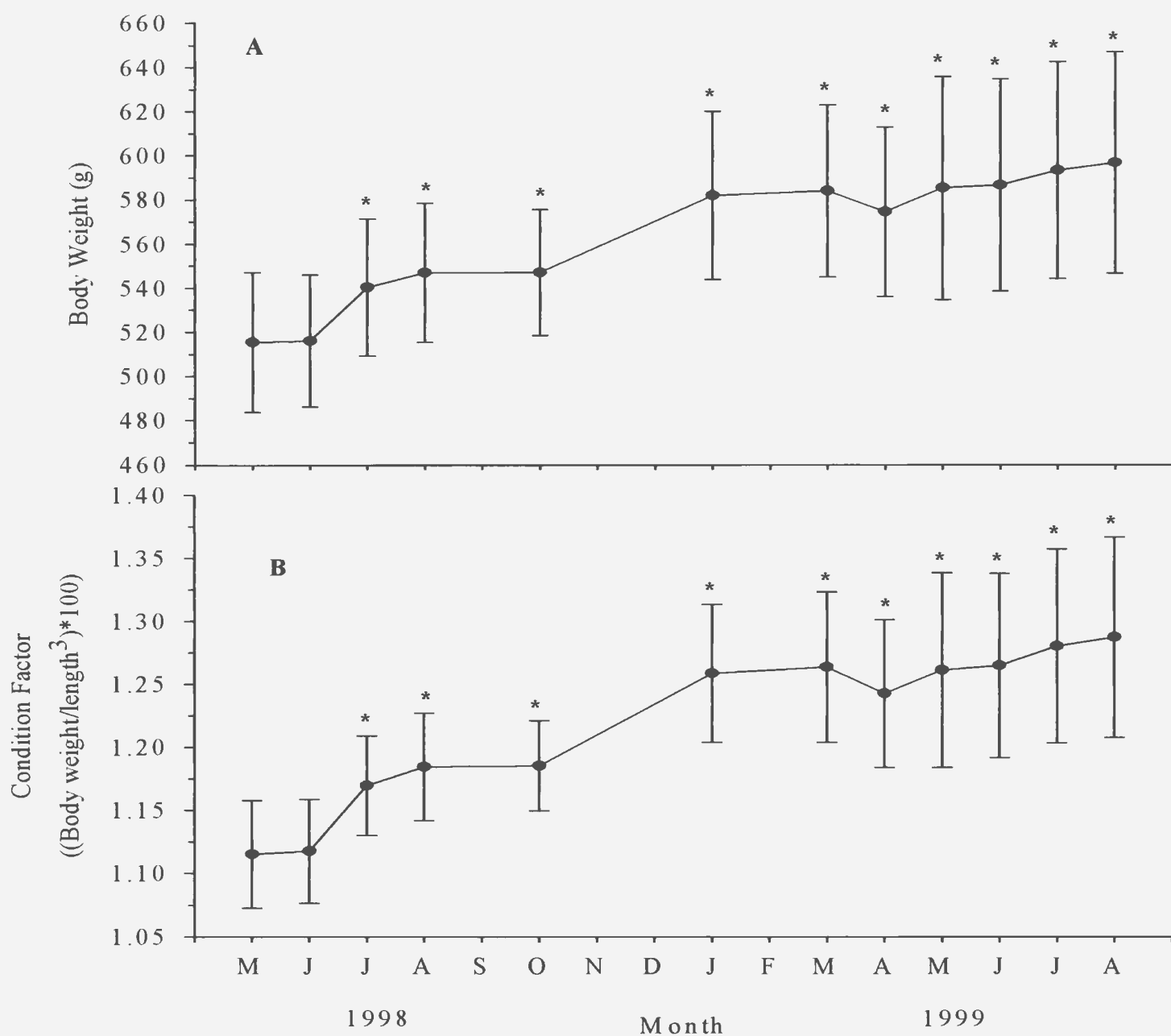


Fig. 2-4: Mean body weight (g) changes (Panel A) and mean CF (%) changes (Panel B) in non-spawning female witch flounder from May 1998 to August 1999 (n=8).

Means with an asterisk are significantly different from the initial time period (Wilcoxon Signed Ranks Test,  $p < 0.05$ ).

**Table 2-2: Daily Egg Volumes (ml) for female witch flounder**

Date	Fish # 1	Fish # 2	Fish # 3	Fish # 4
July 7	-	-	-	-
July 9	-	-	-	-
July 11	-	-	-	-
July 13	< 1	-	-	-
July 15	75	< 1	-	-
July 17	< 1	35	-	-
July 19	-	< 1	< 1	-
July 21	-	-	58	-
July 23	-	-	3	-
July 25	-	-	-	< 1
July 27		-	-	53
July 28	DNC	-	-	< 1
July 30	DNC	DNC	-	-
August 01	DNC	DNC	-	-
August 03	DNC	DNC	DNC	-
August 05	DNC	DNC	DNC	-
August 07	DNC	DNC	DNC	-

DNC: Did not check

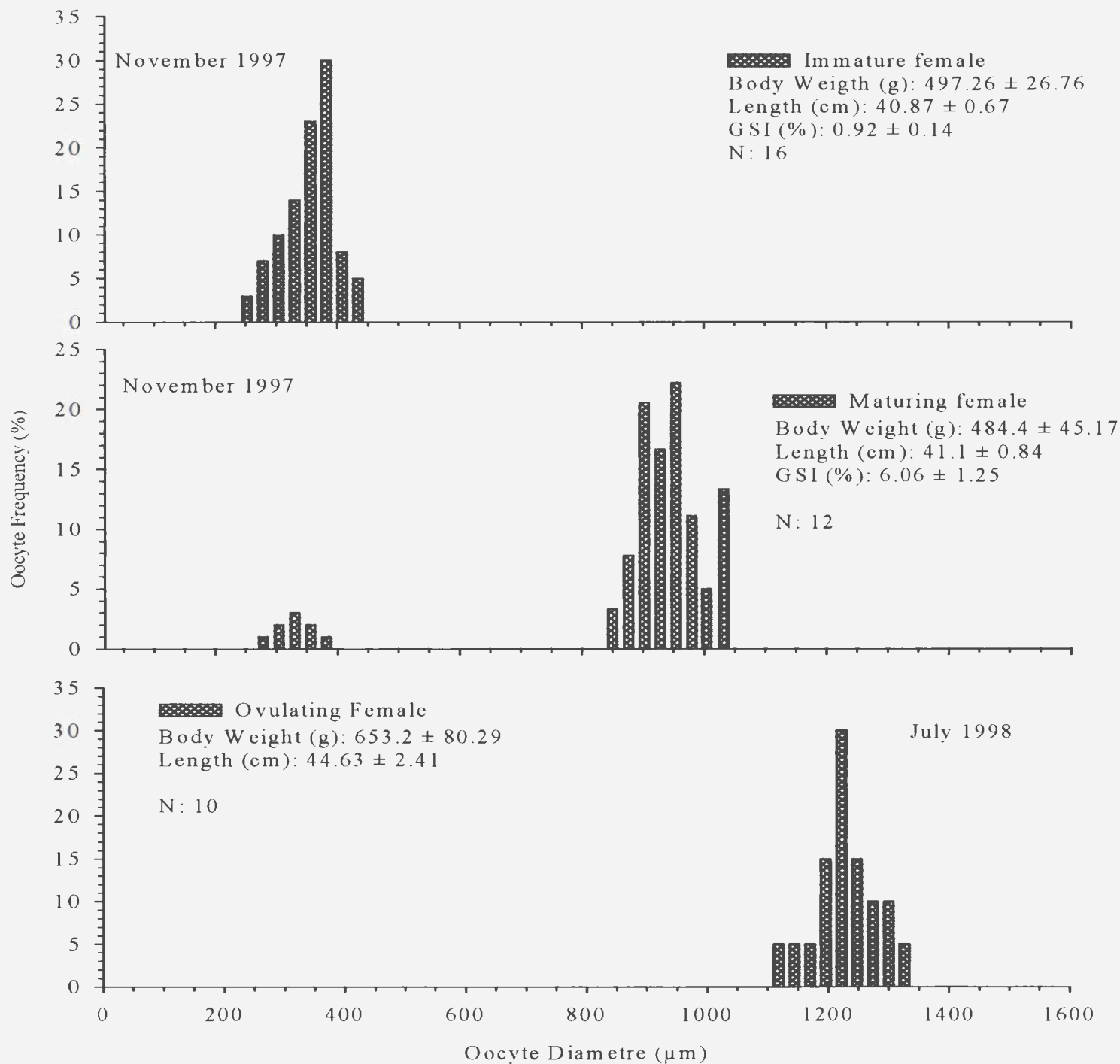


Fig. 2-5: Ovarian biopsies from mortalities after fish collections in November 1997 and July 1998.

#### 2.3.7.4 Hatch Success

Hatch rates fluctuated from 58% to 68%. Numbers reflect an average of three replicates  $\pm$  standard error (Table 2-3).

## 2.4 DISCUSSION

There are several methods to capture witch flounder brood fish- by seines, nets, and angling. The method chosen for a specific species depends on the location depth, and abundance of fish available. This study utilized a seine, which is effective for fishing large areas. This type of gear is relatively good for broodstock collection because it does not drag as much as other gear types. It is generally the most popular, and versatile method of collection. However this method can also cause physical damage to the animal. Animals collected in the initial moments of the tow are subjected to extremes pressures of crowding between the net and subsequent caught animals.

In this study fish were initially caught in November 1997 and was less than successful. From the time the fish were brought on board the trawler, they were carefully handled and maintained with cool, flow through water. The trip to shore lasted one hour. Once on land, fish were transferred to a tank inside a cargo truck. Transport back to the lab was also uneventful with the water quality and temperature carefully monitored. During the transfer to the holding tanks, much physical damage (bruising, small cuts and fin tears as well as scale damage) was noted. This suggests that the method of collection was physically intense and damaging to the fish. Also, these fish were caught at a depth of 260m and the speed at which

**Table 2-3: Egg quality for female witch flounder**

Date	July 15 Fish 1	July 17 Fish 2	July 21 Fish 3	July 27 Fish 4	Average
Body Weight (g)	973	596	895	812	
Egg Volume (ml)	77	37	62	55	55.25 ± 10.48
Total Number of Eggs	55594	26751	44826	39215	
Number of eggs / kg fish	57136	44884	50084	48294	50099.5 ± 2581.57
Viability (%)	82.3 ± 1.20	67.3 ± 1.45	90.0 ± 3.61	80.3 ± 1.76	80.0 ± 2.63
Fertilization Success (%)	88.7 ± 0.88	71.3 ± 1.76	85.3 ± 1.45	81.0 ± 0.58	81.6 ± 2.04
Hatch Success (%)	61.7 ± 4.33	61.0 ± 3.21	68.3 ± 1.76	58.0 ± 3.46	62.3 ± 1.82

the fish were brought to the surface could also be a contributing factor to the heavy mortalities. The duration and speed of the tow as well as the method of bringing the fish to the surface can be altered to minimize the physical injury to the fish.



Additionally, the timing of collection is important. The second collection was attempted in July 1998. This time, the duration and speed of the tow was of a shorter duration to attempt to reduce the physical damage to the animals. However, increased water temperatures and proximity to the spawning season contributed to the mortalities. Water temperatures ranged from 14 - 19°C. All animals returned to the lab ultimately died. It should be noted that all fish collected were mature females that would have spawned in the near future. Animals should not be collected during the spawning season as females are in a particularly delicate condition, and also not during times of seasonally high air and water temperatures. In conclusion, brood fish must be handled carefully to minimize physical injury and stress. Speed and gentleness during fish capture and handling, are of utmost importance. Damage to the slime (mucus) layer, scales and skin of the fish can result in infection. It is known that in at least one species of flatfish the epidermis can undergo profound seasonal changes in thickness and fragility (Burton and Fletcher 1983) and a histological study would indicate whether this is the case for witch flounder. Crowding, dissolved oxygen depletion, rapid changes in temperature and osmotic imbalances are well known causes of stress and must be avoided when transporting fish.

Overall growth for the fish in this experiment was good. Condition factors were higher than expected (ranging from 0.9 - 1.3% in males; 1.1 – 1.3% in non-spawning females; 1.2-1.5% in spawning females) from data collected in the wild. Beecham (1982) reported witch flounder with CF's in the range of 0.94 – 1.09%. This could be attributed to a variation in the diets used for the two groups of fish. Cultured witch flounder have had reported CF's as high as 1.1% in 1-year fish and 1.4% in 4 year fish (Unpublished data, Aquaculture Resource Development Facility, St.John's, NL – D. Boyce 2004). These fish were the offspring of the eggs collected in this experiment and had been reared on commercial diets.

Oocyte size-class frequency distributions of witch flounder demonstrate the presence of just a single clutch of progressively developing vitellogenic oocytes, indicating group-synchronous development, by far the most common of teleost reproductive strategy in teleosts. The homogeneity of oocyte size in the maturing female witch flounder suggest that they are seasonal spawners, like the winter flounder (Harmin et al. 1995), in contrast to species that spawn multiple batches such as halibut, yellowtail (Norberg et al 1991) and the English sole, *Parophrys vetulus* (Johnson et al. 1991). The data reported for egg volume collections also supports the theory of a single seasonal spawning event. While small amount of eggs can be collected around the primary egg release, regular significant volumes of eggs cannot be collected over an extended period as shown in the batch-spawning yellowtail flounder (Manning and Crim 1998).

Good quality eggs are usually defined as those that demonstrate low levels of mortality at fertilization, eying, hatch and first-feeding and those which produce the fastest-growing and healthiest fry and older fish (Bromage et al. 1992). Egg survival and hatching rates, however, while being the ultimate measure of egg quality, tells us nothing about the factors that determine egg quality. There is no general agreement on methods for quality assessment in eggs of marine fish. “Good” quality pelagic eggs are generally distinguished from “poor” quality eggs by virtue of the eggs ability to float in seawater (Brooks et al. 1997).

The quality of the eggs collected from the females in this study was very good. The viability, fertilization and hatch rates were similar to those reported for yellowtail and summer flounder (Manning and Crim 1997; Watanabe et al. 1998).

Two terms are often used to describe the fecundity of fish: absolute fecundity, which is the total number of eggs ovulated per fish, and relative fecundity, which is the number of eggs, ovulated per unit (kg) body weight. The number of eggs is typical of a species, even

though, as with egg size, intraspecific genetic variation, age, body size, environmental conditions and nutrition may increase the variance in this number (Tyler and Sumpter 1996).

Marine teleosts, producing small pelagic eggs, tend to be very fecund. The sole (*Solea solea* L.), for example, has a relative fecundity of 200,000 – 400,000 (Millner et al. 1991). Teleosts with the highest absolute fecundity include cod and halibut, which produce several million eggs each season.

Witch flounder, in the wild, have an average relative fecundity of 300,000 eggs kg<sup>-1</sup> (Bowering 1978). This is considerably greater than the 50,000 eggs kg<sup>-1</sup>, reported in this study. The wild fish reported in this study were considerably larger in length and there is evidence that larger fish produce larger number of eggs and smaller fish produce smaller number of eggs (Kjesbu et al, 1996). Very little research has been done on the genetics of fecundity in fish but it is likely, that as in mammals, there is a strong genetic basis. Putting genetics aside, a number of studies have indicated that fecundity can be modulated by body growth rate and/or nutrition. For example, studies on the winter flounder have shown that there is a nutritionally sensitive period where lack of energy reserves during gametogenesis causes the winter flounder to switch off gonadal development (Burton 1994). Previous studies in captive yellowtail flounder have shown that 50% of the females were not attaining their respective fecundity estimates (Manning and Crim 1998).

None of the eight females in this experiment spawned in 1998 and only 4 spawned in 1999. This could be a reflection of poor holding conditions, stress due-to handling, dietary deficiencies or any number of factors, which can influence reproductive success. However it may be possible that these fish do not spawn every year. The occurrence of non-reproductive individuals has been noted previously in the long rough dab, *Hipploglossoides platessoides*, winter flounder and the European plaice, *Pleuronectes platessa*. It has been suggested that

these fish do not spawn every year once having attained a certain size and age. More importantly, poor condition determines a reproductive strategy where trade-offs are made in egg production to maintain a good body size for a better year (Burton and Idler 1984).

The primary concern of any fish hatchery is to produce the maximum number of high quality eggs from the available broodstock. There is little doubt that poor husbandry profoundly affects the number and quality of the eggs produced in cultured fish (Bromage et al 1992). Ideally broodfish should be maintained under controlled conditions, which as far as possible match or improve upon those to which the fish will have been exposed in the wild. Unfortunately it is almost impossible to manage all of the rearing conditions. Water temperatures and quality, feeding regime and diet, stocking density, handling stress must all be optimized to establish the best husbandry practices for the successful reproductive performance of the broodstock fish.

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## CHAPTER 3.

### **The reproductive steroidal cycle of the witch flounder, *Glyptocephalus cynoglossus*.**

#### **3.1 INTRODUCTION**

In order for reproduction to occur in fish, they have to undergo gonadal development and maturation, and gamete final maturation resulting in ovulation in females (release of eggs into the oviduct) and sperm production in males. Bottlenecks can occur at any of these stages, possibly as a result of inappropriate environmental conditions (social and physical) and/or chronic stress imposed by the conditions of captivity (Pankhurst, 1998). Controlled approaches to managed reproduction rely on a solid understanding of the pattern of gamete development, duration and frequency of spawning events and associated endocrine changes. Armed with this information, potential bottlenecks can be identified and management strategies implemented.

Witch flounder, *Glyptocephalus cynoglossus*, has never previously been held in captivity throughout the annual cycle of reproduction, therefore there is no information on whether they will mature or produce viable gametes. Levels of reproductive steroids in the blood have been used in a number of fish species as indicators of reproductive development (e.g. Scott et al 1984 and Scott and Canario 1990) and should be useful in elucidating the mechanisms involved in reproduction of the witch flounder.

This study examines the seasonal changes in reproductive steroids found in the blood of captive witch flounder; focusing on the plasma levels of the gonadal steroids testosterone (T), 17 $\beta$ -estradiol (E<sub>2</sub>), and 11-ketotestosterone (11-KT). These steroids were chosen for measurement because they are markers of reproductive events in other teleosts species.

E2 and T are commonly measured in females as indicators of ovarian development (Pankhurst and Carragher 1991; Methven et al 1992) while in male teleosts, elevated levels of plasma T are often associated with spermatogenesis (Fostier et al 1987; Pankhurst and Conroy 1987; Harmin et al 1995). 11KT is also suggested to play a role in spermatogenesis (Borg 1994; Harmin et al 1995) and possibly spermiation (Fostier et al 1987; Carolsfield et al 1996).

## **3.2 MATERIALS AND METHODS**

### ***3.2.1 Experimental fish***

In August 1998, twelve mature females and six mature males, from the captive broodstock, were selected for this study. Females were selected by the observance of vitellogenic oocytes (yellow in color- indicating the presence of yolk) in the ovary. Oocytes were gently aspirated from the anterior of the right (eyed side) ovary by inserting a polyethylene cannula with an inner diameter of 1.9mm through the ovipore (Larsson et al 1997). Gentle pressure was applied to the abdomen of the fish and males were identified by the presence of milt from the urogenital pore. Fish were held in the same tanks and conditions as described in Chapter 2.

### ***3.2.2 Blood collection***

During the experimental period, in addition to the monthly measurements, these fish were not anaesthetised and were restrained by hand, during which a blood sample was collected. Using a pre-heparinized syringe with a 23G needle, a 0.5ml blood sample was taken from the

caudal vein, transferred to a 1.5ml micro-centrifuge tube and then stored on crushed ice for 1-2 hours. The blood was then centrifuged for 10 minutes, 8325 x g at 4°C (Heraeus Sepatech, Contrifuge 17RS, Germany). Blood plasma was aliquoted and stored in 0.5ml micro-centrifuge tubes at -70°C until sex steroid radioimmunoassay analysis.

### ***3.2.3 Radioimmunoassay***

For determination of 11-ketotestosterone concentrations (11-KT), ether was used to extract steroids from a 50µl sample of plasma and prepared for RIA according to Harmin and Crim (1993). The extraction efficiency ranged from 87-95% and all data were corrected accordingly. The 11-KT was assayed using a tritiated label synthesized from tritiated cortisol as described by Truscott (1981). The antibody was diluted at 1:50,000, and tritiated 11-KT, ca. 5000cpm, were added to each tube. 11-KT antibody cross-reactivity with testosterone and 11β-hydroxy-testosterone was <0.1% (Ng and Idler, 1980).

Testosterone (T), in both male and female plasma, and 17β-estradiol (E2) concentrations in female plasma were determined using a no-extraction, solid-phase <sup>125</sup>I radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). The Coat-A-Count procedure is a solid-phase radioimmunoassay, based on steroid-specific antibody immobilized to the wall of a polypropylene tube. A 100µl of plasma was pipetted directly into the bottom of the antibody-coated tubes (in duplicate). After the addition of 1.0 ml of <sup>125</sup>I-labelled steroid the tubes were vortexed and then incubated at room temperature. This is where <sup>125</sup>I-labelled steroid competes with the steroid in the plasma sample for antibody sites on the tubes. After a 3-hour incubation at room temperature, the separation of bound from free is achieved by

decanting. The tubes containing the bound fraction are then counted for 1 minute in a gamma counter (Packard Autogamma 5650 Series, United Technologies Packard), with the counts being inversely related to the amount of steroid present in the sample. The quantity of steroid in the sample is determined by comparing the counts to a calibration curve.

T antibody cross-reactivity was non-detectable for cortisone, estradiol, estrone and progesterone, 0.01% for androstendione, and 0.027% for 11-KT. E2 antibody cross-reactivity was 0.32% for estriol, 10.0% for estrone, 0.006% for 11-KT, 0.001% for testosterone and was non-detectable for progesterone (Instruction manual, DPC, 1999).

Intra-assay (within assay) variation for the male androgens testosterone and 11- KT was 4.3% (n=5) and 6.2% (n=5), respectively. Intra-assay (within assay) variation for testosterone, and 17 $\beta$ -estradiol, in females was 5.7% (n=5) and 4.3% (n=5), respectively. All samples were done within one assay so inter-assay (between assays) variation could be eliminated.

#### ***3.2.4 Statistics***

Data were analyzed using one or two way ANOVA and Duncan's multiple range means test ( $P < 0.05$ ) (Sokal and Rohlf, 1969) on the statistical computer package, SPSS. Data were log, square root or arcsine transformed when necessary to fit the assumptions of ANOVA. Data are expressed as means  $\pm$  standard error.

### 3.3 RESULTS

#### 3.3.1 Females

Of the twelve females chosen for this experiment, only four spawned. For this reason, the steroid profiles were separated for spawning and non-spawning females.

Spawning females showed seasonal changes in both the plasma  $E_2$  ( $F = 4.544$   $p < 0.05$ ) and T ( $F = 2.528$ ,  $p < 0.05$ ), (Fig. 3-1). Plasma  $E_2$  began to elevate in January or earlier, being 4.3 ng/ml compared with 2.4 ng/ml in August 1998. and steadily increased to and decreased sharply to 2.1 ng/ml in August. Plasma T ranged between 3.2-4.7 ng/ml until increasing rapidly in May to 8.5 ng/ml and decreased in August.

Non-spawning females showed a seasonal increase in  $E_2$  ( $F = 2.528$ ,  $p < 0.05$ ) during the spawning season but no seasonal change in T ( $F = 0.079$ ,  $P = 0.608$ ) (Fig. 3-2). Similar to the spawning females, but with slightly lower overall levels, plasma  $E_2$  ranged between 2.5 and 3.5 ng/ml until slight increases in May to 4.0 ng/ml, in June to 4.98 ng/ml and to 6.2 ng/ml in July and finally decreases in August. Plasma T remained constant from 2.2 to 3.2 ng/ml.

#### 3.3.2 Males

Males showed seasonal changes in both plasma T ( $F = 3.498$ ,  $p < 0.05$ ), and 11-KT ( $F = 4.209$ ,  $p < 0.05$ ) (Fig. 3-3). Plasma T ranged from 2.25 to 3.25 ng/ml from October to March when sperm was not present and then increased to 4.5 ng/ml at onset of visible milt production, peaked in June to 11.5 ng/ml and decreased rapidly to 2.25 ng/ml in August. Plasma 11-KT ranged between 1.75 to 3.15 ng/ml from October to March,

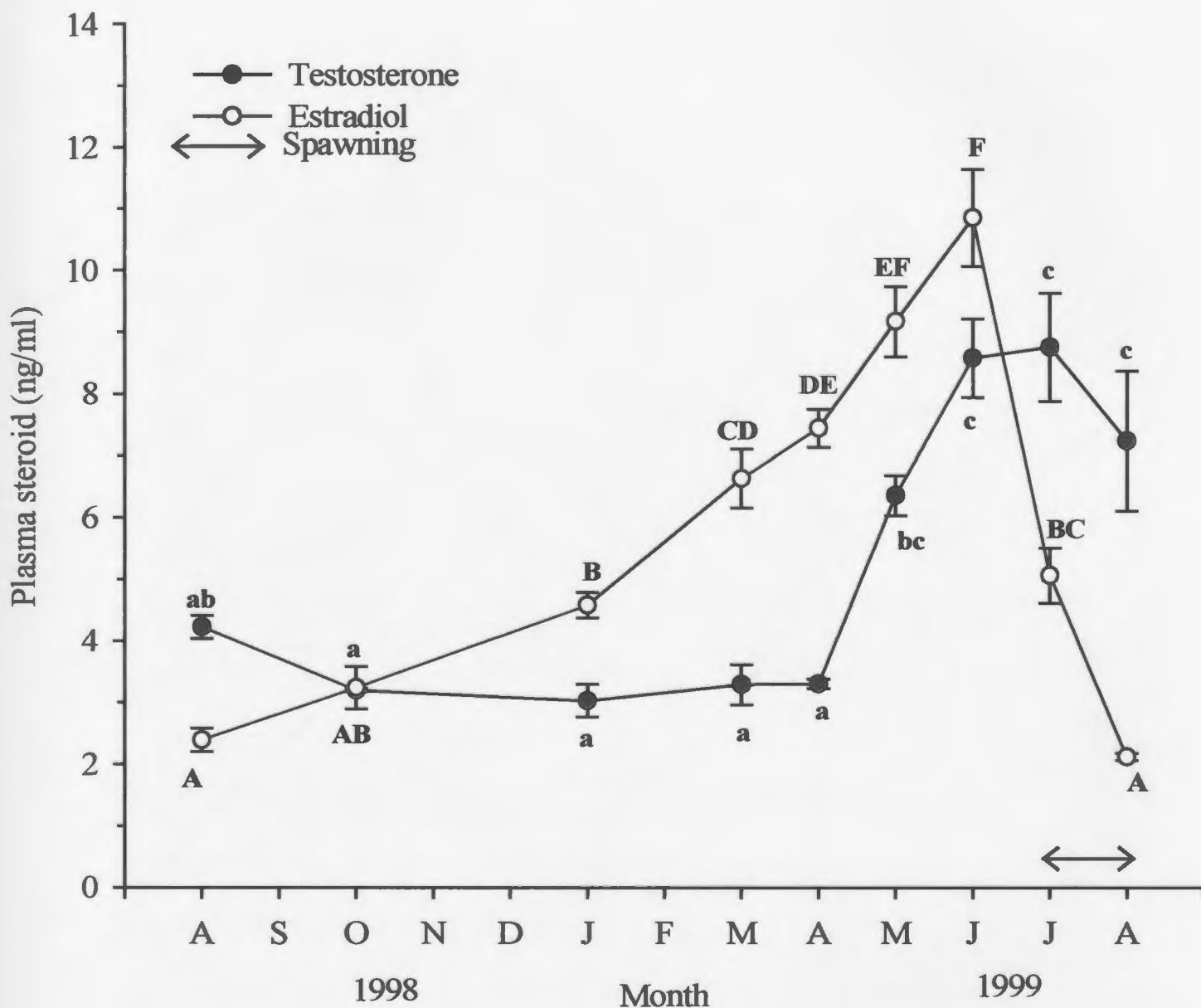


Fig. 3-1: Seasonal changes in plasma steroids (ng/ml) for spawning female witch flounder, *Glyptocephalus cynoglossus*.

Means  $\pm$  standard error  
plasma E2 (empty circles) and plasma T (filled circles)

Means with similar letters were not significantly different (Duncan's multiple range test,  $p < 0.05$ ). Upper and lower case letters show Duncan's grouping of plasma E2 and T respectively.

increased in to 7.25 ng/ml in April and peaked in May and June at 12.25 ng/ml then decreased to 4.5 ng/ml in July and 2.25 ng/ml August.

### 3.4 DISCUSSION

The reproductive cycle of both the male and female witch flounder is characterized by distinct seasonal variations and fluctuations in plasma sex steroids associated with reproductive activity.

The role of steroids T and E<sub>2</sub> is well established in several teleost species. T is a precursor for the synthesis of E<sub>2</sub> (Matsuyama et al 1988) and is also involved in positive feedback stimulation of the pituitary synthesis of gonadotropin (GtH) (Crim et al 1981; Barnette and Pankhurst 1999). E<sub>2</sub> stimulates synthesis of the yolk precursor vitellogenin, which is incorporated into the growing oocyte during vitellogenesis (Specker and Sullivan 1993). As vitellogenesis approaches completion there is a fall in E<sub>2</sub>, then T and a surge in the production of 17 $\alpha$ 20 $\beta$ -dihydroxy-4-pregen-3-one, which is the maturation steroid in most species in which it has been investigated (Scott and Canario, 1987). The annual cycles of plasma T and E<sub>2</sub> have been reported in the female greenback flounder *Rhombosolea tapirina* (Gunther) (Barnett and Pankhurst 1999), plaice *Pleuronectes platessa* L. (Wingfield and Grimm, 1977, Scott et al 1998), halibut *Hippoglossus hippoglossus* L. (Methven et al 1992), yellowtail flounder *Pleuronectes ferrugineus* (Clearwater 1996) and winter flounder *Pleuronectes americanus* (Walbaum) (Harmin et al 1995).

While the elevated plasma levels of E<sub>2</sub> and T in the female witch flounder that released viable eggs were consistent with changes in plasma T and E<sub>2</sub> reported in other species, the

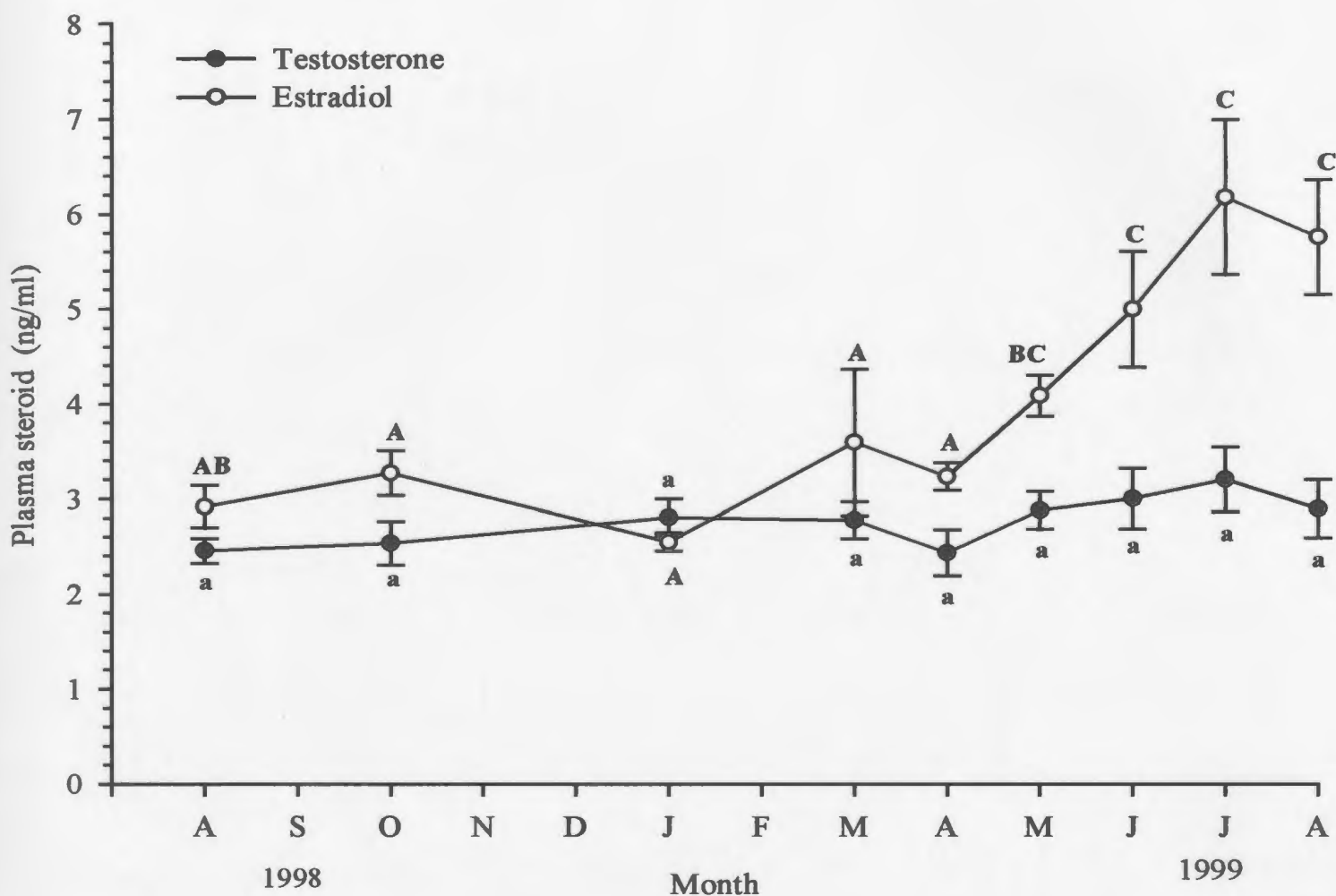


Fig. 3-2: Seasonal changes in plasma steroids (ng/ml) for non-spawning female witch flounder, *Glyptocephalus cynoglossus*. Means  $\pm$  standard error. Plasma E2 (empty circles), plasma T (filled circles)

Means with similar letters were not significantly different (Duncan's multiple range test,  $P < 0.05$ ). Upper and lower case letters show Duncan's grouping of plasma E2 and T respectively.



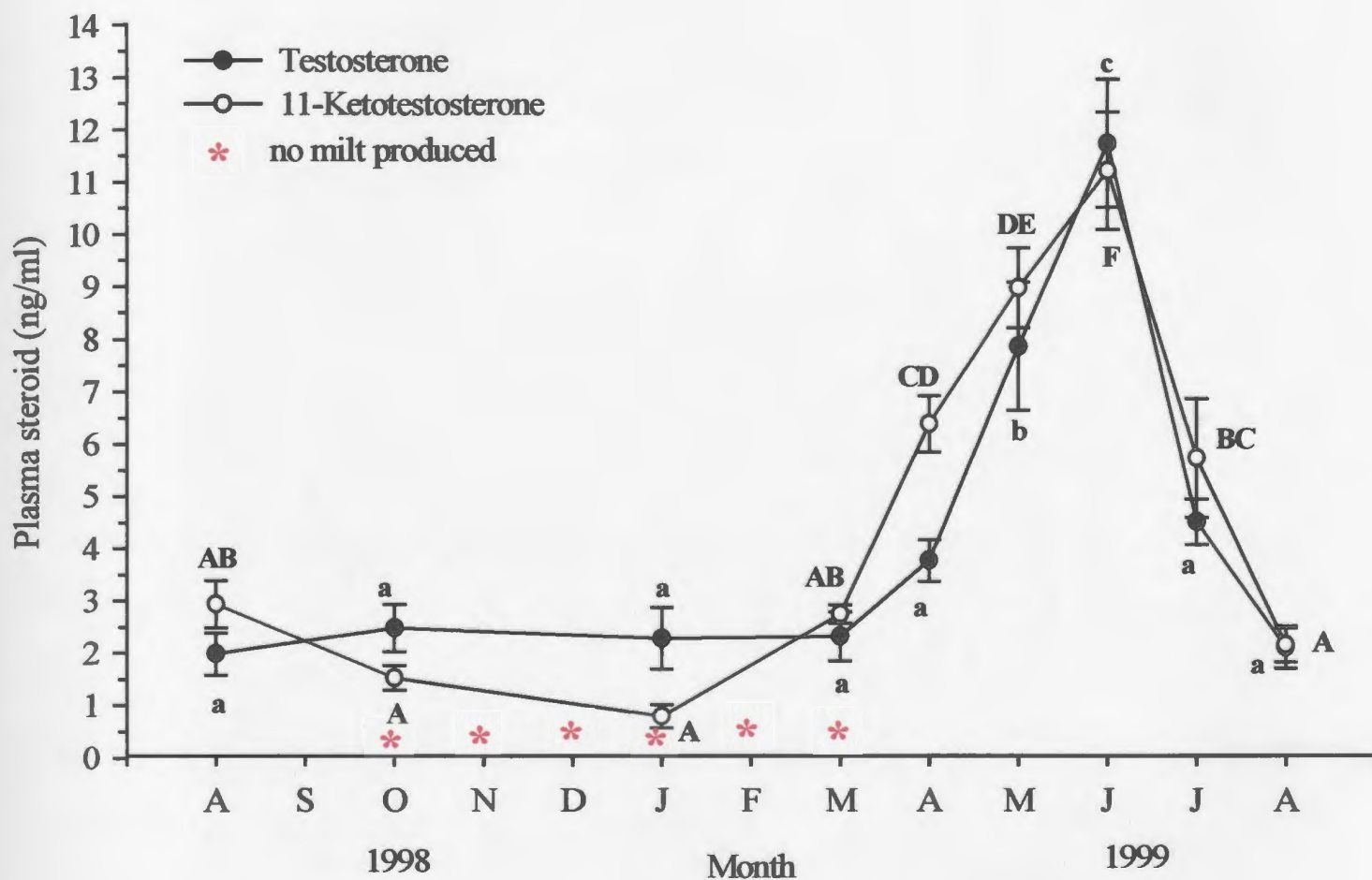


Fig. 3-3: Seasonal changes in plasma steroids (ng/ml) for male witch flounder, *Glyptocephalus cynoglossus*. Means  $\pm$  standard error. 11-KT (empty circles), plasma T (filled circles)

Means with similar letters were not significantly different (Duncan's multiple range test,  $p < 0.05$ ). Upper and lower case letters show Duncan's grouping of plasma 11-KT and T respectively.

range of steroids found the witch flounder are more consistent with lower steroid levels (<25ng/ml) found in the yellowtail and greenback flounder than the halibut and winter flounder which generally have steroid levels greater than 25ng/ml (Harmin et al., 1995; Methven et al. 1992). The levels of plasma E<sub>2</sub> were low in August and began to increase throughout the winter season and peaked in late June, before the beginning of the spawning season in July and then decreased rapidly. Plasma T levels remained low until April and then increased rapidly to peak at the beginning of the spawning season and decreased until the end of the spawning season in August.

The non-spawning females showed a similar but lower seasonal plasma E<sub>2</sub> cycle, however there was no seasonal change in plasma T. The physiological role of T in female teleost reproduction is not well understood; evidence from other species suggests that this pattern of seasonal change could be related to the role of T as a precursor to E<sub>2</sub> (Kagawa et al. 1983) or to its possible role in stimulating the pituitary release of GtH in the pre-spawning GtH surge (Young et al 1983, Kobayashi et al. 1989). Testosterone may also be of importance to oocyte maturation and ovulation in coho salmon (Fitzpatrick et al 1987). It should be noted that T levels in spawning females didn't increase until well after plasma E<sub>2</sub> began to increase. Since all females showed the presence of vitellogenic oocytes in the ovary at the beginning of this study, without the seasonal increase of T in this group, females failed to undergo ovulation, suggesting that the role T may be important to these functions. Since none of these fish produced eggs in 1998 (see Chapter 2), it is also possible that these fish do not spawn every year but could take much longer between reproductive events.

The annual reproductive cycle of the male witch flounder is accompanied by fluctuations in plasma sex steroids similar to the changes recorded in females. Following the end of a spawning season, plasma T and 11-KT levels are very low, similar to levels reported in other

marine species such as winter flounder (Harmin et al 1995), plaice (Scott et al 1998), greenback flounder (Barnette and Pankhurst 1999) and halibut (Methven et al 1992). Thereafter, androgen levels remained relatively steady until the spawning period when maximum concentrations of T and 11-KT were observed in spermiating males.

In male teleosts, plasma T levels tend to be highest during spermatogenesis (transition from spermtogonia to spermatids) and tend to drop off prior to spermiation (sperm release) (Methven et al 1992, Harmin et al., 1995; Carolsfield et al., 1996, Barnette and Pankhurst 1999). In many species 11-KT is elevated during spermatogenesis and the early stages of spermiation, and is thought to be more effective than T at stimulating spermatogenesis, secondary sexual characteristics and stimulating behaviour (Borg 1994).

Plasma T and 11-KT generally began to increase prior to spermiation and dropped before the onset of spermiation in many species such as halibut, winter and greenback flounder. In this study, I found that T is low preceding milt production, increasing with the beginning of spermiation and the highest concentrations were detected in the middle of the spermiation period; 11-KT is elevated coincidently with T in these fish.

In summary, this study is the first to describe the endocrine changes associated with reproduction in the witch flounder. As seen in other marine teleosts, the circulating levels of sex steroids increased as gamete maturation and gonad growth proceed, reaching peak levels during spawning.

This work will provide a framework upon which strategies for controlling witch flounder reproduction can be designed. Concentration can now be focused on over coming the hurdle of incomplete final oocyte maturation and ovulation in the broodstock females.

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## CHAPTER 4.

### **Spermiation and milt characteristics of the male witch flounder (*Glyptocephalus cynoglossus*) in captivity.**

#### **4.1 INTRODUCTION**

Like with many externally fertilizing marine teleosts, witch flounder (*Glyptocephalus cynoglossus*) sperm is immotile in the seminal plasma and when collected from the urogenital pore (Morisawa and Susuki, 1980). During natural reproduction, motility is induced upon contact with seawater and mass forward motility tends to last from 30-120 seconds. The particular factors that suppress motility are, as a rule, neutralized by the environmental conditions during spawning. Therefore, in teleost fish, sperm activation is thought to be triggered by changes in osmotic pressure, pH and ionic composition of the diluent compared to the seminal plasma (Billard et al. 1992). In marine fish, an increase in osmotic pressure relative to the seminal plasma is the most commonly known factor causing sperm activation, with motility being less sensitive to changes in pH (Billard et al. 1992)

Various traits are used to assess reproductive condition and sperm quality in fish. These include sperm motility, adenosine triphosphate (ATP) concentration, sperm density, gonadosomatic index and various components of the seminal plasma. Many of these traits have been examined to devise techniques for short-term storage, long-term cryopreservation or for comparative studies (Geffen and Evans 2000).

Witch flounder males produce relatively low volumes of highly concentrated sperm (200-350 $\mu$ l) for five months of the year, beginning in April and finishing in September. This



correlates with the timing of ovulation in the females. In order to manage male witch flounder broodstock effectively it is important to have a basic understanding of sperm availability, quantity and quality as well as factors such as pH, osmolality, which influence sperm activation. With this knowledge, further work can be done on improving sperm volume and possibly sperm storage since the limited sperm volume could be a potential problem for an aquaculture operation.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 *Experimental fish***

The same six males, from the previous study (Chapter 3), were followed from May 1998 through August 1999 for this study. Males were identified by the presence of milt from the urogenital pore, after applying gentle pressure to the abdomen of the fish. Milt was only available during five months of the year (April through August). However, in 1998 there were no samples collected in April. The fish were held in the same tanks and under identical conditions as the fish described in Chapter 2.

### **4.2.2 *Milt collection***

For milt collection, the males were carefully dried and all available milt was manually stripped from the urogenital pore in a pre-chilled tuberculin syringe and stored in 0.5ml micro-centrifuge tubes for analysis. Urine contamination was unavoidable at times, but its

presence/absence was noted. For both years, milt was collected once a month during the spermiation period.

#### **4.2.3 Motility**

Approximately 1 hour after milt collection, motility was checked by making a 1:10 dilution (one part semen: 9 parts diluent) using a substitute seminal plasma, containing 150mM sucrose, plasma 7mM MgSO<sub>4</sub>, 1.7mM CaCl<sub>2</sub>, 86mM glycine, and 30mM Trizma (pH 8.0) (Billard et al., 1993). A 10µl pipette tip was dipped into the diluted sperm sample and quickly stirred into a 100µl sample of seawater already prepared on a chilled microscope slide on the stage of the microscope (Micromaster, Fisher Scientific, Model CK). Percentage of sperm clearly demonstrating forward motion at the point of mixing was recorded for each sample. The data for percentage of sperm activated were grouped into arbitrarily defined classes (Table 4-1). Motility estimates were replicated three times for each sample with a blind procedure.

#### **4.2.4 Milt Volume**

Milt volume was measured using a 1.0ml Nichyio pipette (Fisher Scientific). Milt was repeatedly drawn up in the pipet until the entire sample was measured without any air bubbles. Measurements were recorded to the nearest 10µl.

#### 4.2.5 Milt pH

Milt pH was measured by dripping milt from a glass pipet, directly on indicator paper (ColorpHast sticks, EM Science, New Jersey, U.S.A.; pH range 6.5-10.0). pH was measured and recorded twice for each sample.

**Table 4-1: Ranking of sperm motility**

Motility	Rank
No motile sperm	0
<25% motile sperm	1
26 – 50 % motile sperm	2
51 – 75% motile sperm	3
76-100% motile sperm	4

#### 4.2.6 Spermatocrit

Spermatocrit (packed cell volume) was determined, in duplicate, on small aliquots of milt in sealed 70 $\mu$ l capillary tubes (ID 1.1 to 1.2 mm; length 75mm, Pyrex Corning). After centrifuging (Hematocrit Microcentrifuge, International Equipment Co., Model MB) at 15,000 x g for 30 min., the packed cell level and total volume was measured to the nearest 0.5mm.

The spermatocrit value calculation was determined as follows: % spermatocrit = packed cell

(mm) / total volume of milt (mm) x 100 (Baynes and Scott, 1985). Three replicates of undiluted milt in haematocrit tubes were measured for each sample.

#### **4.2.7 *Seminal Plasma Osmolality***

Seminal plasma was obtained by the centrifuging of milt samples, in 0.5ml micro-centrifuge tubes, for 10 minutes, 8325 x g at 4°C. Seminal plasma was removed from the top of the packed cells, stored in tubes and placed on ice. Osmolality was then determined by using a Fiske 110 osmometer (Fiske Associates, Massachusetts, USA). Three 10µl aliquots were measured and the results averaged to obtain the osmolality of each seminal plasma sample.

#### **4.2.8 *Statistical Analysis***

Data were analyzed using non-parametric Wilcoxon Signed Ranks Test ( $p < 0.05$ ) using the SPSS computer package. Data were log, square root or arcsine transformed when necessary to fit the assumptions of normality. Data are expressed as means  $\pm$  standard error.

### **4.3 RESULTS**

#### **4.3.1 *Motility (Rank)***

Sperm motility varied from the beginning of the spermiation season until the end of the spermiation period in August (Fig. 4-1). There was no significant difference in motility

rankings between 1998 and 1999 ( $p < 0.495$ ). There was no significant seasonal pattern within the rankings in 1998 ( $p > 0.352$ ) or in 1999 ( $p < 0.117$ ). However, there was a significant difference between July and August in both years. Motility was lower at the beginning and end of the period of spermiation.

#### **4.3.2 Milt volume**

Milt volume ranged between 250-325 $\mu$ ls (Fig. 4-2). There was no significant difference in milt volumes in 1998 and 1999 ( $p > 0.576$ ). There was also no significant seasonal effect on milt volumes for 1998 ( $p > 0.676$ ) or 1999 ( $p > 0.240$ ).

#### **4.3.3 Milt pH**

At the initiation of spermiation, the mean pH of the milt varied from 7.3 in 1998 and 7.1 in 1999. The milt pH increased in July to 7.6 for both years and then declined to 7.1 at the cessation of spermiation in 1998 and 7.3 in 1999 (Fig. 4-3). There was a significant difference in the milt pH from 1998 and 1999 ( $p > 0.015$ ) and there was a significant seasonal influence on the milt pH ( $p > 0.002$ ) in 1998 but not in 1999 ( $p > 0.244$ ).

#### **4.3.4 Spermatocrit**

At the beginning of the spermiation season, the spermatocrit ranged from 56-65% and gradually increased to 74% and declined to 65% at the conclusion of the spermiation period

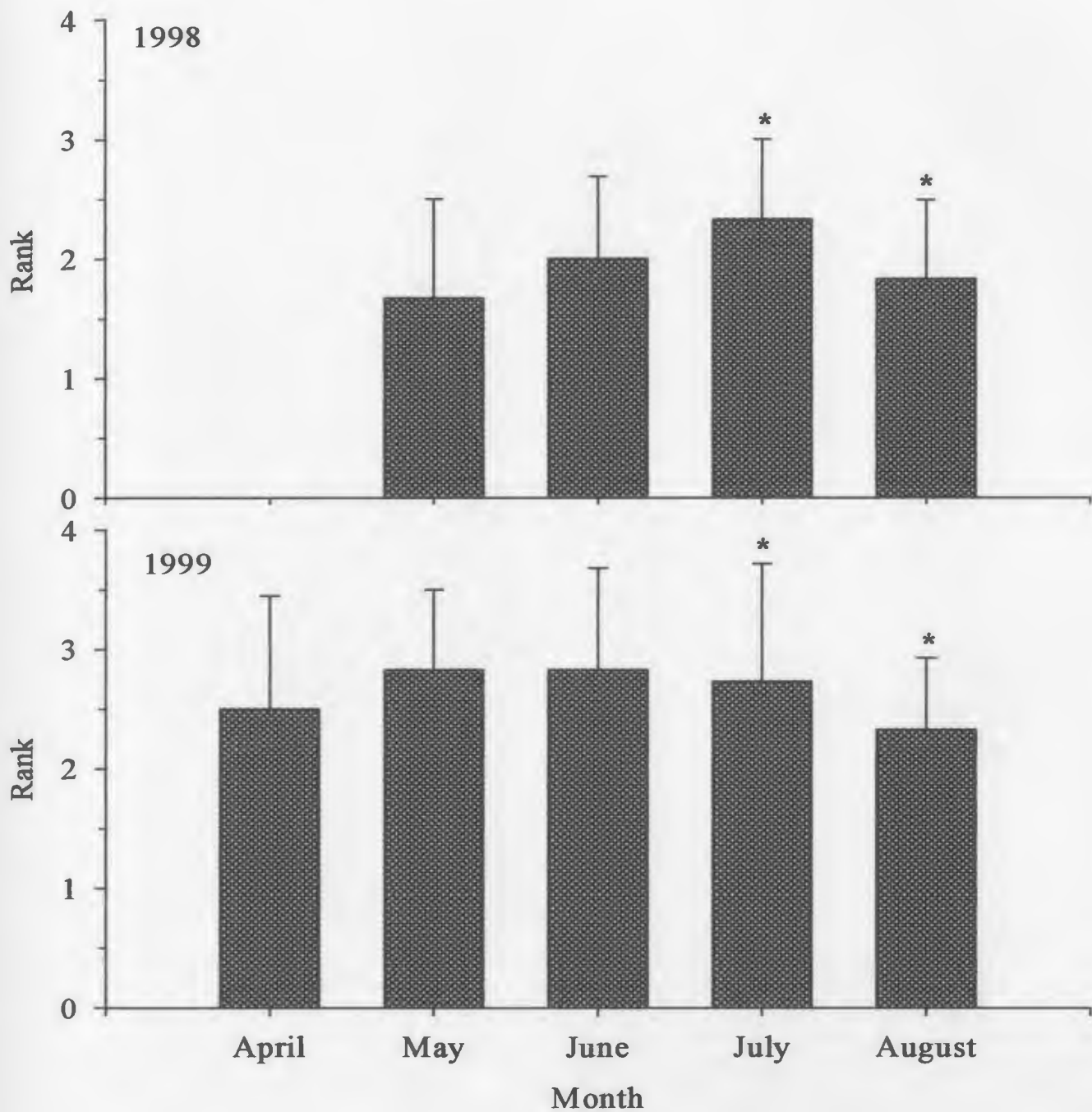


Fig. 4-1: Mean seasonal motility rankings for male witch flounder sperm. Means  $\pm$  standard error

Means with an asterisk are significantly different from each other (Wilcoxin Signed Ranks Test,  $p < 0.05$ ).

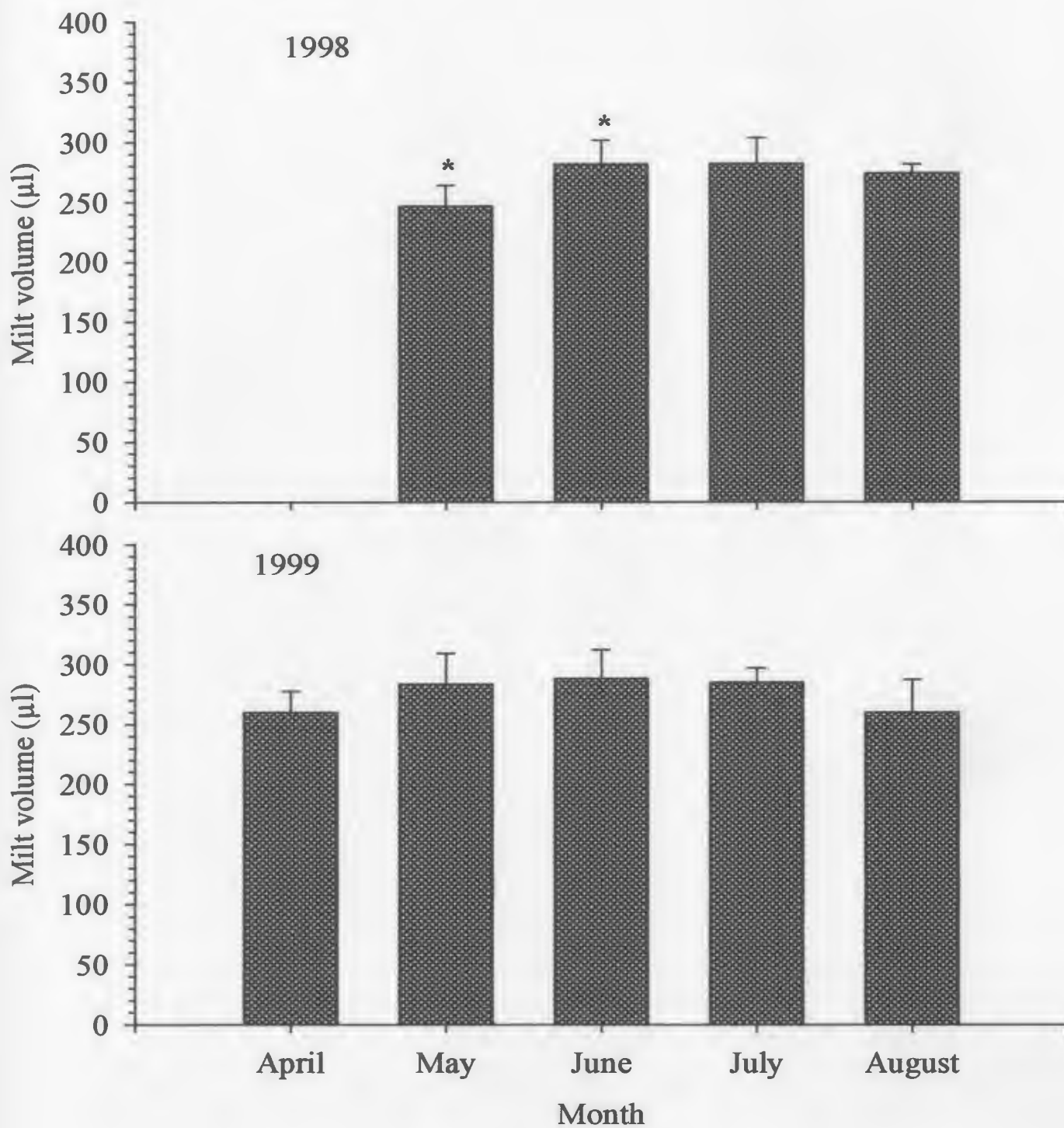


Fig. 4-2: Mean seasonal changes in milt volume (to the nearest 10μl) of the male witch flounder, *Glyptocephalus cynoglossus*. Means  $\pm$  standard error

Means with similar symbols were not significantly different (Duncan's multiple range test,  $p < 0.05$ ).

(Fig. 4-4). There was no significant difference in spermatocrit between 1998 and 1999 ( $p>0.168$ ). There was also no significant seasonal influence on spermatocrit for either year ( $p>0.079$  and  $p>0.110$  respectively).

#### **4.3.5 Seminal Plasma Osmolality**

The osmolality of the seminal plasma ranged from  $325 \text{ mmol kg}^{-1}$  in May 1998 and April 1999, increased to  $330 \text{ mmol kg}^{-1}$  in July and decreased to  $327 \text{ mmol kg}^{-1}$  in August (Fig. 4-5). There was a significant difference in seminal plasma osmolality in 1998 and 1999 ( $p>0.020$ ). There was no significant seasonal influence on seminal plasma osmolality in 1998 ( $p>0.123$ ), however there was a significant seasonal influence in 1999 ( $p>0.028$ ).

#### **4.4 DISCUSSION**

Generally, duration of sperm motility in teleosts is relatively short and mature males approach females and release spermatozoa immediately after oviposition (Morisawa and Suzuki 1980). The released sperm can reach the spawned oocytes within a short period. It is speculated that during the approach of sperm to oocyte, shrinkage and maybe swelling of sperm cells caused by changes in external osmolality, causes the initiation of motility of the motility apparatus in the flagellum, resulting in sperm motility in teleosts (Takai and Morisawa 1995).

Motility is the most commonly used parameter to evaluate sperm quality, although fertilization capacity remains the most conclusive (Coward et al 2002). This parameter is acceptable, as in general sperm must be motile to achieve fertilization. Evaluation of motility



requires some care. A high dilution is required (at least 1:1000) to initiate synchronously the motility of all the sperm. Several methods are used to measure motility. As in this study, the most commonly used in the past was the estimation of the global movement of the sperm according to an arbitrary scale, usually 0 to 5 units (Billard et al 1992). Motility of witch flounder sperm does not appear to be influenced by the time of spermiation. The motility remains unchanged from the initial point of spermiation until sperm can no longer be collected.

Male witch flounder produce low volumes ( $0.6 - 0.8 \text{ ml kg}^{-1}$ ) of viscous milt (Sct 60-80%) similar to yellowtail flounder, *Pleuronectes ferrugineus* (Clearwater and Crim 1998). Sperm is only available for five months of the year (April – August). This correlates with spawning events in the female witch flounder, with ovulated eggs available from late June to late August. Small amounts of milt are difficult to collect and handle, particularly if the milt is highly concentrated and viscous, also urine contamination which can have a negative effect on sperm quality, is difficult to avoid (Clearwater and Crim 1996). It is well established that the stimulation of milt production, in many teleosts, can be achieved by treatment with gonadotropin (GtH) or gonadotropin releasing hormone (GnRH) (Pankhurst 1994). Future work should include investigation into use of this treatment to stimulate both sperm production and milt volume.

Increase in pH of milt is also a factor in sperm motility in sea urchins (Christen et al 1982) and in mammals (Babcock et al 1983). Motility of witch flounder sperm was best in July when the mean pH ranged between 7.5-8.0 (Fig 4-6).

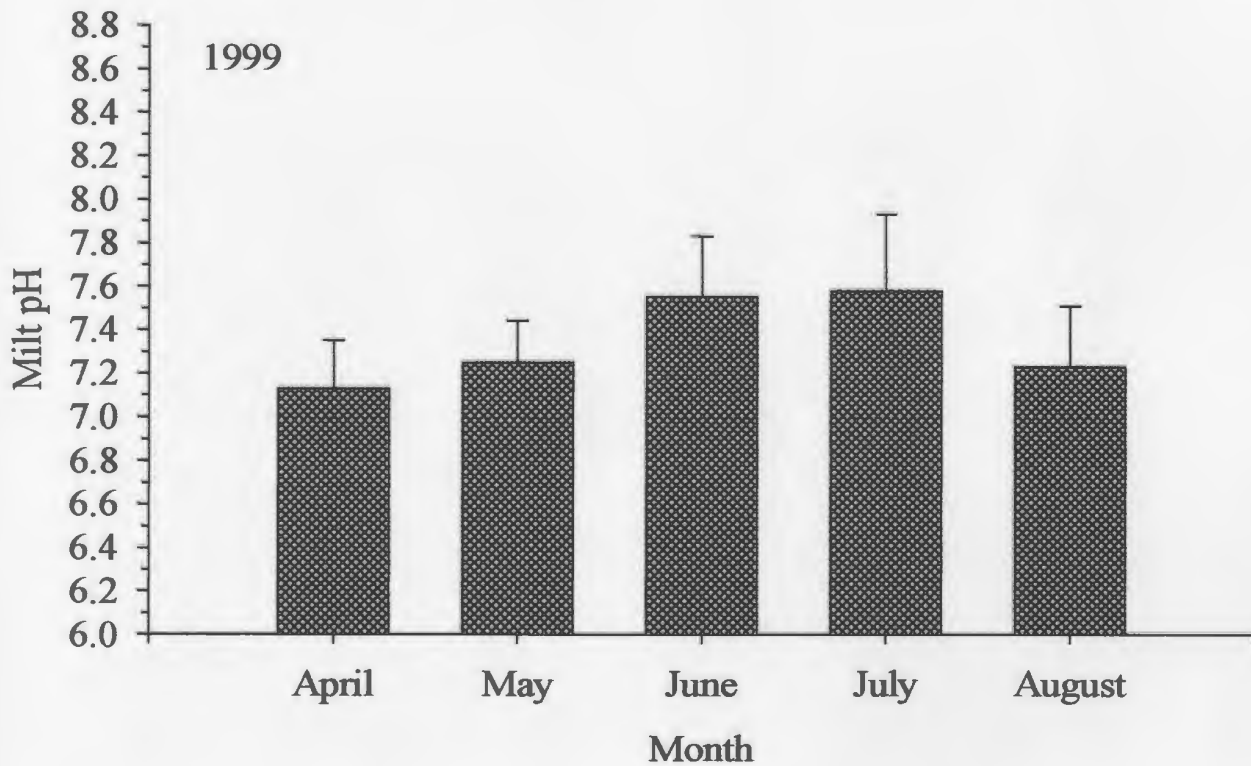
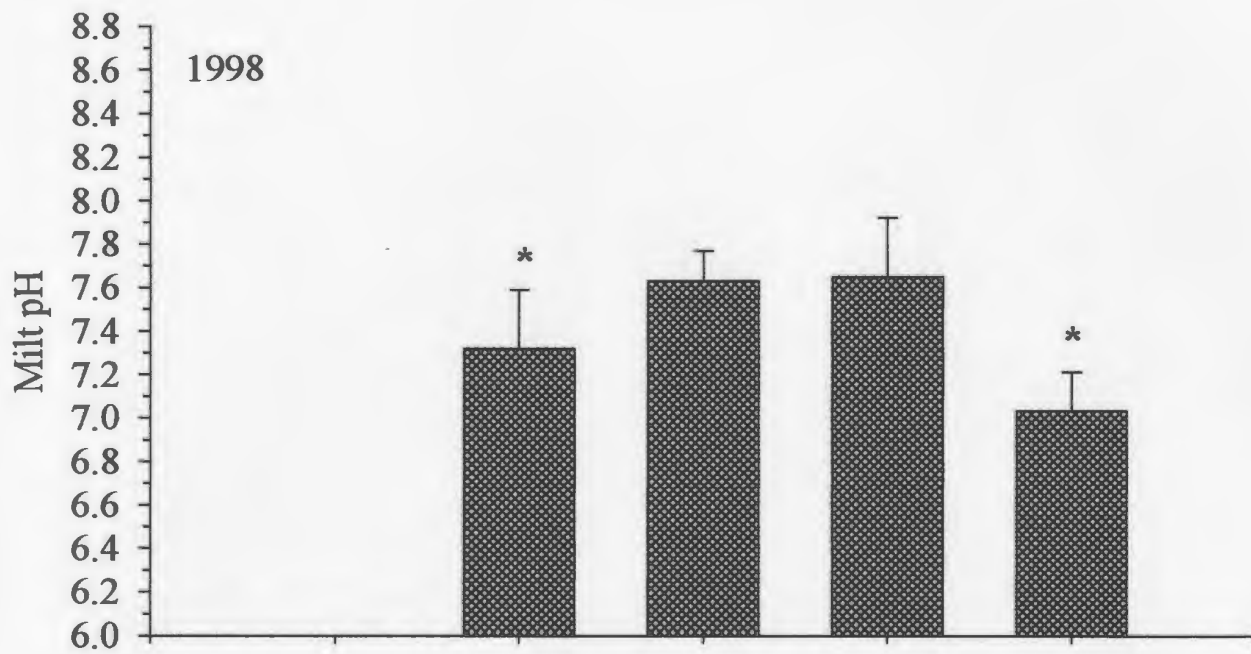


Fig. 4-3: Mean seasonal milt pH for male witch flounder, *Glyptocephalus cynoglossus*. Means  $\pm$  standard error

Means with an asterisk are significantly different from each other (Wilcocon Signed Ranks Test,  $p < 0.05$ ).

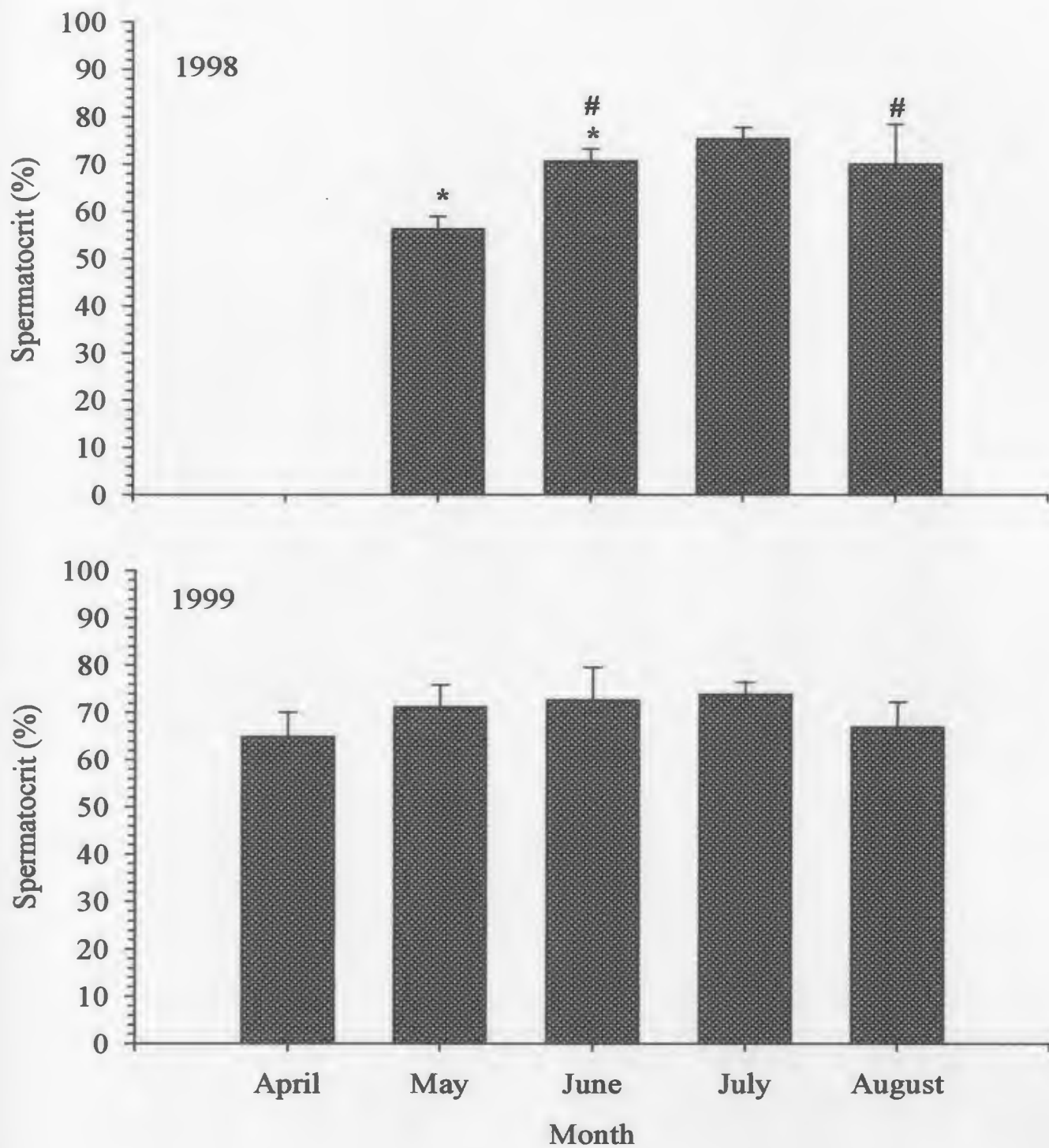


Fig. 4-4: Mean seasonal changes in percent spermatocrit (%) of the male witch flounder, *Glyptocephalus cynoglossus*. Means  $\pm$  standard error

Means with matching symbols are significantly different from each other (Wilcoxon Signed Ranks Test,  $p < 0.05$ ).

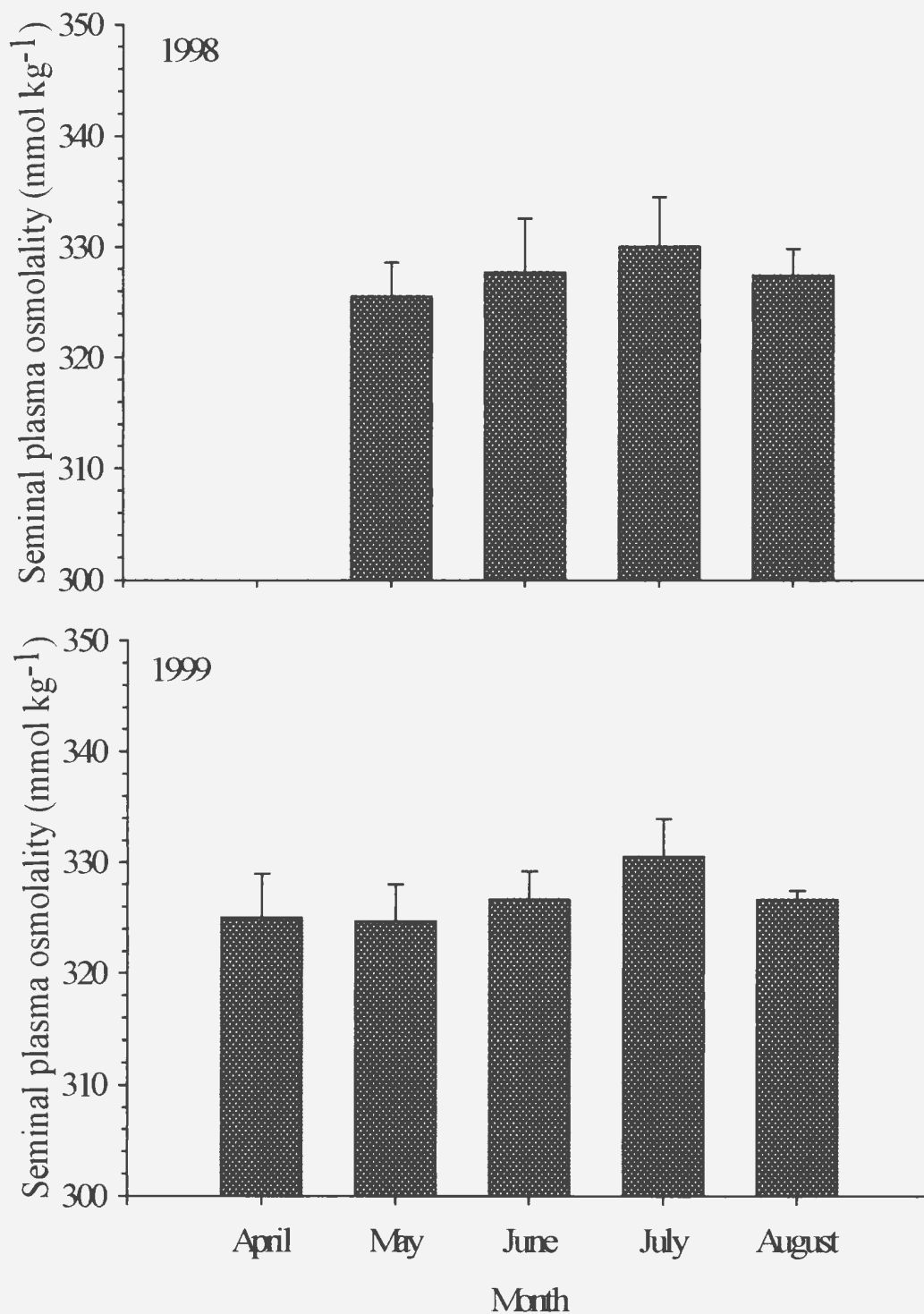


Fig. 4-5: Mean seasonal changes in osmolality of the seminal plasma (mmol kg<sup>-1</sup>) of the male witch flounder, *Glyptocephalus cynoglossus*. Means  $\pm$  standard error

Means with similar symbols were significantly different (Wilcoxon Signed Ranks Test,  $p < 0.05$ ).

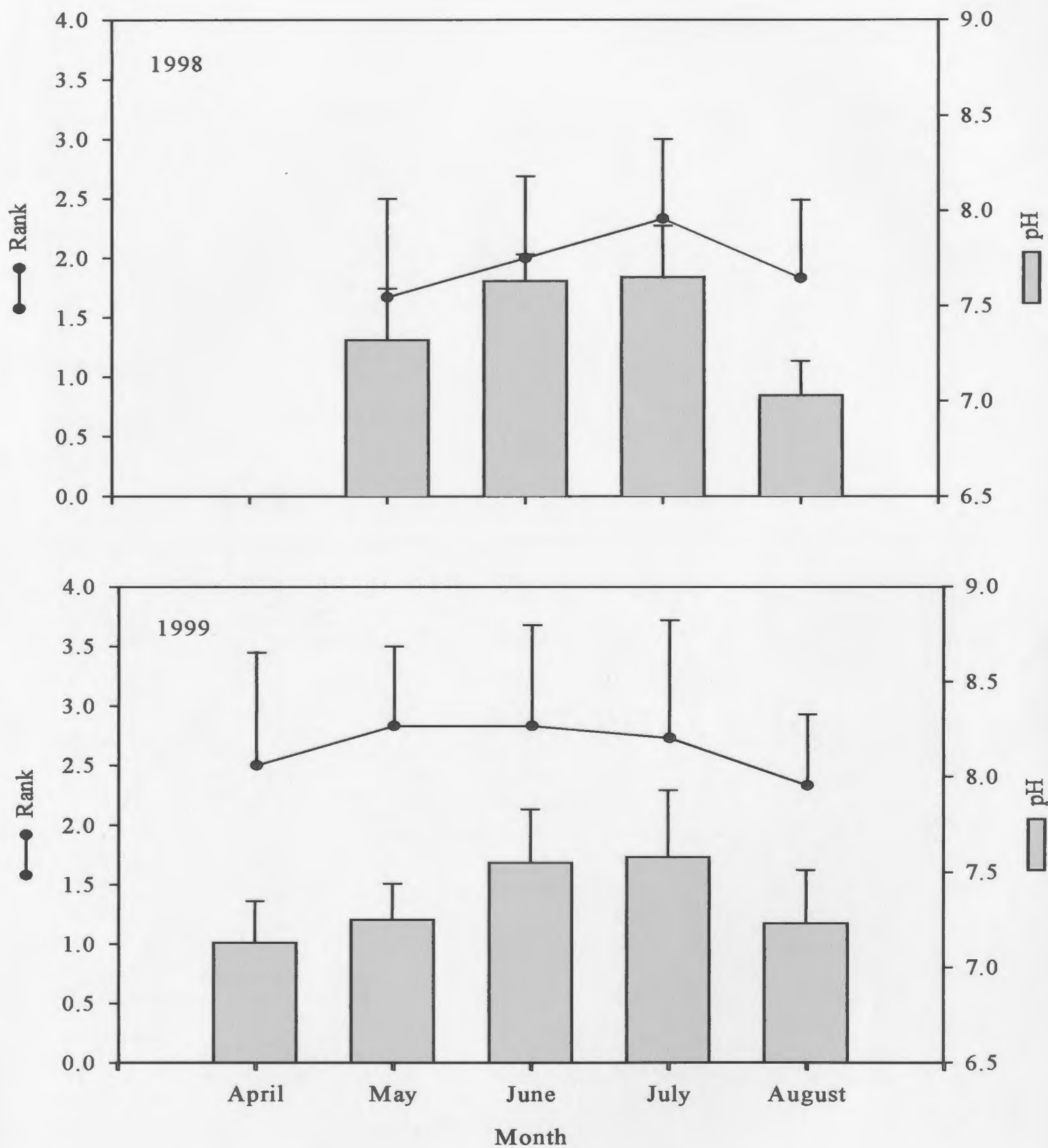


Fig. 4-6: Relationship between sperm motility (means  $\pm$  standard error) and milt pH (means  $\pm$  standard error) in male witch flounder, *Glyptocephalus cynoglossus* over the spawning season.

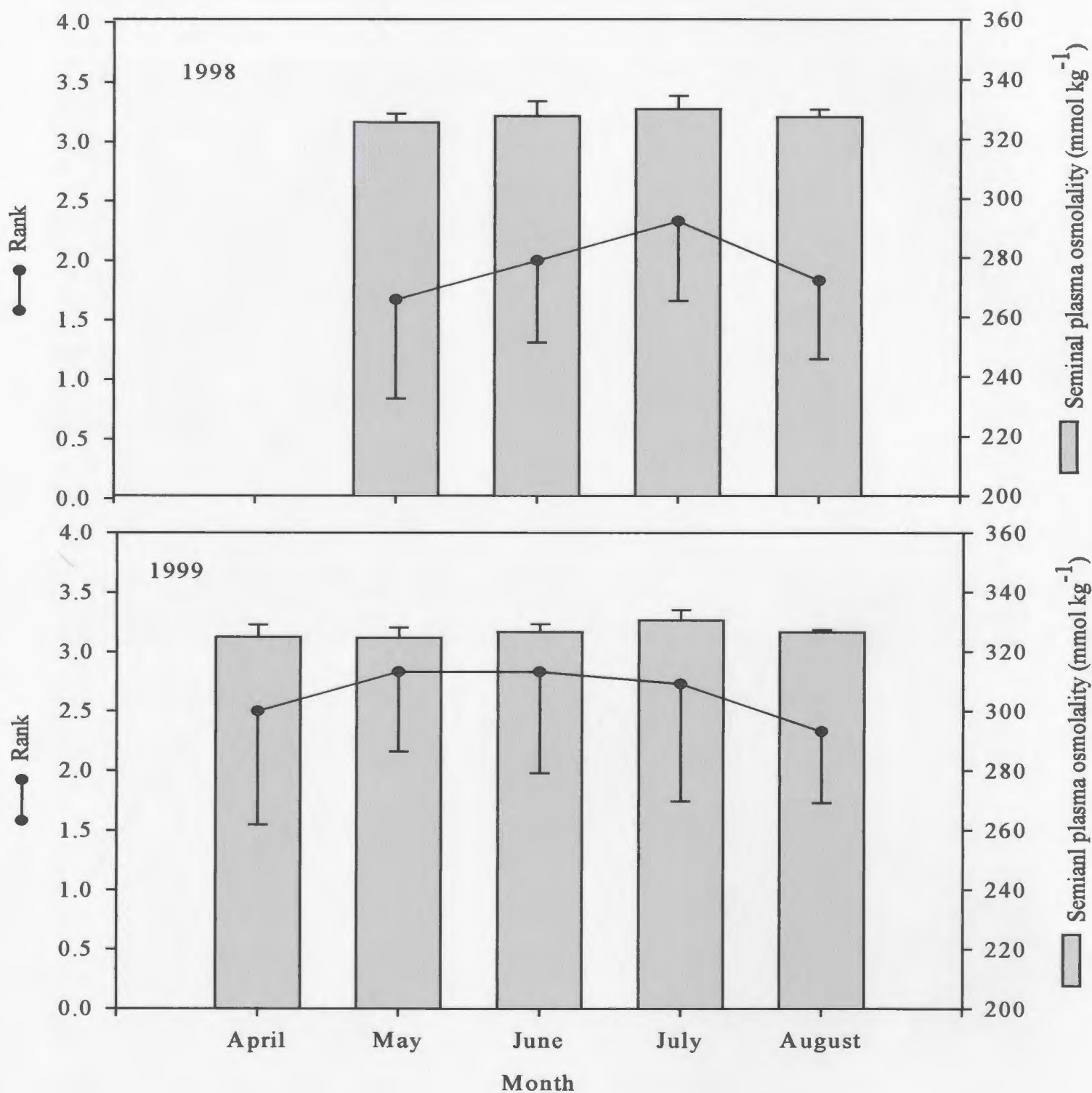


Fig. 4-7: Relationship between sperm motility (means  $\pm$  standard error) and seminal plasma osmolality (means  $\pm$  standard error) in male witch flounder, *Glyptocephalus cynoglossus* over the spawning season.

Seminal plasma osmolality is generally found to range from 240-450 mmol kg<sup>-1</sup> for various freshwater teleosts such as 300 mmol kg<sup>-1</sup> in salmonids and 254-335 mmol kg<sup>-1</sup> in cyprinids (Billard and Cosson 1992) and some marine species e.g. 306 mmol kg<sup>-1</sup> in turbot (Suquet et al 1993), 355.6 mmol kg<sup>-1</sup> in ocean pout (Wang and Crim 1997) and 364.6 mmol kg<sup>-1</sup> in the seminal plasma of the sea bream, *Sparus aurata* (Chambeyron and Zohar 1990, Morisawa 1985). The seminal plasma osmolality in the witch flounder, which ranged between 320-330 mmol kg<sup>-1</sup>, is low among marine fishes, similar to turbot.

A better understanding of sperm biochemistry and physiology and the mechanisms that regulate sperm motility in teleosts, in particular, the mechanisms for the initiation of motility are important for the development of techniques for artificial fertilization in each species. These techniques include short-term preservation and cryopreservation. My study provides a baseline of milt characteristics for the male witch flounder. As a result of this study, further research must be done on improving sperm volumes as well. Low sperm volumes as well as the short duration of spermiation are two areas, which would be of major concern in an aquaculture facility.

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## **CHAPTER 5.**

### **Summary and Future Research**

#### **5.1 SUMMARY**

The experiments described in this thesis focused on developing a captive broodstock and collecting preliminary information about the reproductive strategies, behaviour and endocrinology of the witch flounder. The objective of the experiments was twofold. First, the results can be used to design methods for the capture and holding conditions for optimal growth and maturation. Second, the reproductive information can be used as a both a baseline and guidelines for further studies.

The initial objective was to capture witch flounder and transport them to the Ocean Science Center. Once acclimated to laboratory conditions and feeding on a formulated diet, growth and maturation in the surviving fish was monitored. The lack of success in the capture and collection, overwhelmingly indicate that capture method is extremely important. Timing (season) of collection attempts is also extremely important. After the initial two-week acclimation period, the rate of survival among the remaining fish was very good, with the only mortalities during the study being the post-spawned females. Further research should investigate other methods of fish collection/capture as well as varying tow speed and time with the original collection method.

The overall growth of the witch flounder broodstock was good but highly variable. The weight of male fish increased greater than either group of females. The males grew 54% during the duration of this study, while spawning females increased only 12% and the non-spawning females 13.5%. More work should be done understanding the dietary requirements for optimal growth and reproductive success within the broodstock population.

Male witch flounder produce milt for only five months of the year and this timing is synchronized with the spawning season of the females. The analysis of milt indicates that the motility of witch flounder is highest in July when the seminal plasma pH ranged from 7.5-8.0 and osmolality ranged from 320-330 mmol kg<sup>-1</sup>. The most significant result from this study was the discovery of the low volumes of available milt. Witch flounder males produce low volumes (0.6 – 0.8 ml kg<sup>-1</sup>), viscous milt (Sct 60-80%). Further research must be done on extending the spermiation period as well as increasing overall milt volume.

Female witch flounder demonstrate group-synchronous oocyte development, with only one spawning event per season. Only 30% of the females in this study matured. This could indicate that the diet was insufficient for gonadal development and oocyte maturation or it could also suggest that female witch flounder may not release oocytes every year. All females in this experiment revealed the presence of vitellogenic oocytes in their ovaries at the beginning of the study but still failed to undergo final oocyte maturation and ovulation.

Egg quality in the witch flounder was variable but still very respectable. Viability ranged between 67-90%, fertilization ranged from 71-88% and hatch rates ranged between 58-68%. Future research should include a longer and more intense study of oocyte development. Ovarian biopsies from mature females over several reproductive seasons would give a clearer picture of the pattern of oocyte development as well allow for a closer correlation with the information collected from the plasma steroid analysis.

The reproductive steroids of the witch flounder follow the same seasonal pattern as many other species. For most of the year, circulating levels of androgens, in males, remain low until there is a sharp increase in both plasma T and 11-KT which coincides with the onset of spermiation. The spawning females also demonstrated a clear seasonal cycle in the circulating levels of E<sub>2</sub> and T. Both steroids remain low, with E<sub>2</sub> increasing slightly in advance of T, until

both steroids peak and then drop at the time of ovulation. In the females that failed to spawn, the E<sub>2</sub> levels still reflected a seasonal pattern, however, the levels of T remained low and constant. Further research should investigate the possibly of inducing final oocyte maturation and ovulation through artificial means such as hormone treatments or photoperiod manipulation.

## **5.2 FUTURE RESEARCH**

1. This species acclimates easily to an aquaculture situation if it can be collected with minimal physical damage. One possible suggestion is to reduce towing times and therefore number of animals collected. This could reduce crowding stress during capture and therefore reduce mortality.
2. The time of year for collection can also have an impact on mortality. Increased seasonal water temperatures, seasonal maturation state as well possibly seasonal thinning of skin can influence the survival success at capture.
3. These fish will grow in captivity however, more research on the dietary requirements of these fish is necessary for optimal growth.
4. Female witch flounder will mature and produce viable eggs in captivity but further investigation is required into understanding ovarian and oocyte development.
5. Male witch flounder produce low volumes of milt and future research should concentrate on improving sperm volumes through hormone (GnRH) treatments.







